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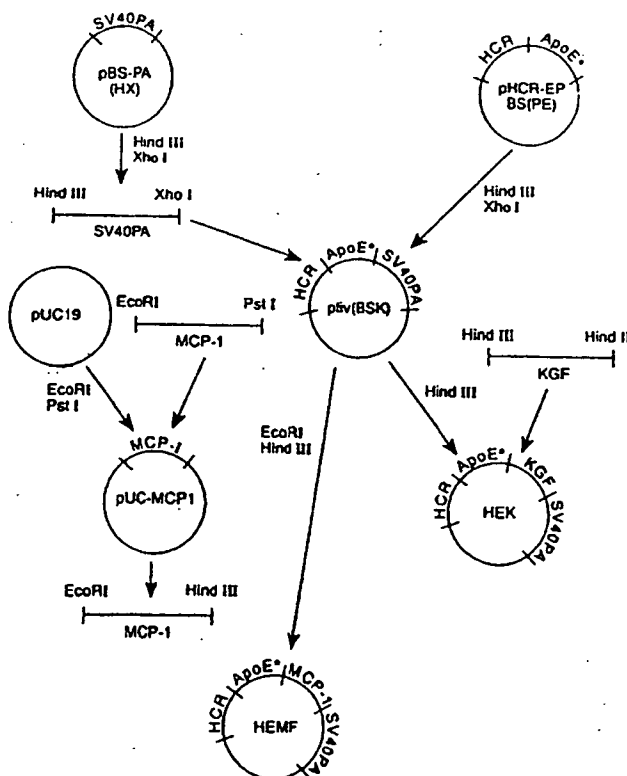
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(54) Title: MAMMAL WITH ENHANCED LIVER EXPRESSION OF A TRANSGENE

(57) Abstract

This invention provides a mammal with enhanced liver expression of a transgene. Also provided are: 1) a nucleic acid sequence useful in enhancing liver specific expression of a transgene, and 2) a vector containing this nucleic acid sequence.



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- 1 -

MAMMAL WITH ENHANCED LIVER EXPRESSION OF A TRANSGENE.

This application is a continuation-in-part of U.S.S.N. 08/141,322 filed October 18, 1993.

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BACKGROUNDFIELD OF THE INVENTION

10 This invention relates to the field of recombinant DNA technology, especially to nucleic acid sequences useful for constructing a transgenic mammal. More specifically, the invention concerns expression of a transgene in certain tissues or organs of a mammal.

15

DESCRIPTION OF RELATED ART1. Tissue Specific Expression

20 Production of a transgenic mammal involves the insertion of a nucleic acid sequence, often called a transgene, which codes for a particular polypeptide, into one or more chromosomes of the mammal. This is typically accomplished by inserting the transgene into
25 the pronucleus of an isolated mammalian egg. The transgene becomes incorporated into the DNA of the developing embryo. This embryo is then implanted into a surrogate host for the duration of gestation. The offspring of the surrogate host are evaluated for the
30 presence of the transgene.

 Expression of the transgene, i.e., production of the protein encoded by the transgene nucleic acid sequence, may confer a new phenotype on the mammal. Depending on the transgene(s) inserted into the animal
35 and the level of expression of the transgene in the mammal, the mammal may become more or less susceptible

- 2 -

to a particular disease or series of diseases. Such transgenic mammals are valuable for *in vivo* screening and testing of compounds that may be useful in treating or preventing the disease(s), and/or for developing methods useful in diagnosing the disease.

While methods for insertion of a novel gene into a mammal have developed rapidly, several problems with the application of this technology remain. One such problem concerns limiting expression of the gene primarily to a selected tissue or tissues where expression is desired.

Enhanced and/or specific expression of a gene in a select tissue or tissues of a mammal is complex. Expression of a gene is typically regulated at least in part by a non-coding nucleic acid sequence termed a promoter. The promoter is often located near or adjacent to the nucleic acid sequence encoding the polypeptide to be expressed. Frequently, the activity of a promoter is in turn regulated by other nucleic acid sequences termed enhancers and suppressors (also known as silencers). Enhancers increase the level of expression of the gene while suppressors or silencers decrease expression. The location of enhancers and suppressors along a nucleic acid sequence with respect to the promoter and coding sequence is quite varied for different genes. Enhancers and suppressors may be located near or adjacent to the promoter, *i.e.*, within about 1 kilobase (kb) along a strand of DNA (chromosome or vector), or may be located at a much greater distance, *e.g.*, up to 50 kb or more away from the promoter on a chromosome and still exert an effect on the activity of the promoter. Further, they may be located upstream (*i.e.*, 5' to the promoter and coding sequence), or downstream (3' to the promoter and coding sequence). Such positioning for promoter activity is a function of both the type of promoter and the type of

- 3 -

enhancer or suppressor used. To further complicate the regulation, enhancers and suppressors may exert their effect on the promoter of more than one gene within a chromosomal locus.

5 Several enhancers and suppressors have been identified. For example, the level of expression of the gene encoding transthyretin is affected by an enhancer element located about 2 kb upstream from the promoter (Yan et al., *EMBO J.*, 9:869-AFM8 [1990]). Liver
10 specific expression of the albumin gene is regulated by an enhancer located about 10 kb upstream of its promoter (Hammer et al., *Science*, 235:53-58 [1987]). Tissue specific regulation of the alpha-fetoprotein gene involves three enhancer elements located 1 to 7 kb
15 upstream of the transcription start site of the gene (Pinkert et al., *Genes & Dev.*, 1:268-276 [1987]).

 Another enhancer is the hepatocyte-specific control region, or "HCR". The human HCR is believed to be about 774 base pairs (bp) in size or less (Simonet et al., *J. Biol. Chem.*, 268: 8221-8229 [1993]), but has
20 recently been reported to be at least somewhat active as a 150 to 154 bp fragment (Breslow, *Proc. Natl. Acad. Sci. USA*, 90:8314-8318 [1993]; Shacter et al., *J. Lipid Res.*, 34:1699-1707 [1993]). The HCR is located on
25 chromosome 19, about 18 kilobases (kb) downstream of the apolipoprotein E (apoE) promoter/gene sequence, about 9 kilobases downstream of the apolipoprotein C-I (apoC-I) promoter/gene sequence, and about 2 kilobases (kb) upstream of the apolipoprotein C-I (apoC-I') pseudogene
30 sequence (Simonet et al., [1993], *supra*; Simonet et al., *J. Biol. Chem.*, 266:8651-8654 [1991]; Simonet et al., *J. Biol. Chem.*, 265:10809-10812 [1990]; Taylor et al., *Current Opinion in Lipidol.*, 2:73-80 [1991]). The HCR appears to be important in expression of the genes ApoE
35 and ApoC-I in the liver; in its absence, these genes are

- 4 -

not expressed at detectable levels in this tissue (Simonet et al. [1993], *supra*).

The effect of the HCR on a heterologous promoter has been evaluated in transgenic mice. The apolipoprotein A-IV promoter and coding sequence were
5 ligated to a 1.7 kb nucleic acid sequence containing the HCR. Transgenic mice containing this construct had high levels of expression of apolipoprotein A-IV in the liver (Simonet et al., *supra*).

10

2. Interleukin-8

The interleukins are a group of naturally occurring proteins that act as chemical mediators of the
15 differentiation processes for red and white blood cells. One of the interleukins, IL-8 (also known as Neutrophil Activating Peptide-1, or NAP-1), has been shown to be a neutrophil chemoattractant with the ability to activate neutrophils and stimulate the respiratory burst (Colditz
20 et al., *J. Leukocyte Biol.*, 48:129-137 [1990]; Leonard et al., *J. Invest. Derm.*, 96:690-694 [1991]). IL-8 has been termed a proinflammatory cytokine due to its involvement in neutrophil recruitment to sites of acute and chronic inflammation.

25 Zwahlen et al. (*Int. Rev. Exp. Path.*, 34B:22-42 [1993]) describe some effects of IL-8 injected into some rodents. When injected intradermally into rats, IL-8 induced neutrophil infiltration at the site of injection. Intravenous injection of IL-8 into rabbits
30 resulted in neutrophil sequestration in the lungs.

Vogels et al. (*Antimicrobial Agents and Chemotherapy*, 37:276-280 [1993]) describe the effect of administering IL-8 to mice either before or after infection of the mice with three different pathogens.
35 Under certain conditions, administration of IL-8 was

- 5 -

shown to have a detrimental effect on the survival of the mice.

Van Zee et al. (*J. Immunol.*, 148:1746-1752 [1992]) describe administration of IL-8 to baboons. The animals developed neutropenia rapidly after IL-8 administration. This neutropenia is transient and is followed by a marked granulocytosis which persists for as long as IL-8 is present in the circulation.

Burrows et al. (*Ann. NY Acad. Sci.*, 629:422-424 [1991]) show that guinea pigs injected with IL-8 had a higher level of T-lymphocyte and eosinophil accumulation in the lung than did control animals.

3. Keratinocyte Growth Factor

15

Keratinocyte growth factor (KGF) is a mitogen that has been identified as specific for epithelial cells, especially keratinocytes (Rubin et al., *Proc. Natl. Acad. Sci. USA*, 86:802-806 [1989]; Finch et al., *Science*, 245:752-755 [1990]; Marchese et al., *J. Cell Physiol.*, 144:326-332 [1990]). KGF has shown potential for repair of epidermal tissues such as the skin, and epithelial tissues of the digestive tract. The DNA encoding KGF has been cloned and sequenced (PCT 90/08771, published August 9, 1990).

Guo et al. (*EMBO J.*, 12:973-986 [1993]) have prepared a transgenic mouse containing a transgene constructed of the human keratin 14 promoter and the human keratinocyte growth factor gene. The mouse showed a number of phenotypic differences as compared with non-transgenics such as wrinkled skin and reduced hair follicle density.

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- 6 -

4. Monocyte Chemoattractant Protein

Monocyte chemoattractant protein (also known as MCP-1) is a protein that is produced by activated leukocytes in response to certain stimuli. The gene encoding human MCP-1 has been cloned and sequenced (Furutani et al., *Biochem. Biophys. Res. Comm.*, 159:249-255 [1989]; Yoshimura et al., *Chemotactic Cytokines*, Westwood et al., eds. Plenum Press, NY [1991], pp.47-56). MCP-1 serves to attract monocytes to the site of its release, and is believed to be involved in the cellular immune response and in acute tissue injury (Leonard et al., *Immunol. Today*, 11:97-101 [1990]). MCP-1 has been shown to be produced by some tumor cells *in vitro*, and in human metastatic melanomas *in vivo* (Graves et al., *Am J. Pathol.*, 140:9-14 [1992]).

5. Human Afamin

Afamin ("AFM") is a novel protein recently identified in human serum. AFM has a molecular weight of about 87,000 daltons when run on SDS_PAGE, and shares significant homology to members of the albumin family of proteins including vitamin D binding protein (VDB), alpha fetoprotein, and albumin. In addition, AFM has the characteristic pattern of disulfide bonds observed in this family. AFM cDNA has been stably transfected into Chinese hamster ovary cells, and recombinant AFM (rAFM) has been purified from the conditioned culture medium of these cells. Both AFM and rAFM react with a polyclonal antibody that was raised against a synthetic peptide derived from the deduced amino acid sequence of AFM. There is a need in the art to provide *in vivo* systems for evaluating the effects of one or more genes on certain diseases.

- 7 -

Accordingly, it is an object of this invention to provide a mammal containing a nucleic acid construct comprising a transgene, and expressing the transgene, where the mammal may be used as an *in vivo* system to
5 analyze the course of a disease.

It is a further objective to provide a transgene nucleic acid construct and an expression vector that enhance tissue specific expression of a transgene in liver tissue of a transgenic mammal.

10 Other such objects will readily be apparent to one of ordinary skill in the art.

SUMMARY OF THE INVENTION

15 In one aspect, the present invention provides a nucleic acid sequence comprising an HCR enhancer operably linked to a promoter and a transgene. The promoter may be selected from the group of promoters consisting of: ApoA-I, ApoA-II, ApoA-III, ApoA-IV, ApoB-
20 48, ApoB-100, ApoC-I, ApoC-II, ApoC-III, ApoE, albumin, alpha feto protein, PEPCK, transthyretin, SV40, CMV, and TK. The transgene may be selected from the group consisting of: interleukin 1, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6,
25 interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, ENA-78, interferon- α , interferon- β , interferon- γ , granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, stem cell factor,
30 keratinocyte growth factor, MCP1, AFM, and TNF, and fragments thereof.

In one other aspect, the invention provides a non-human mammal and its progeny containing a nucleic acid sequence comprising an HCR enhancer operably linked
35 to a promoter and a transgene.

- 8 -

The invention further provides a non-human transgenic mammal containing nucleic acid sequence comprising an HCR enhancer, the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human IL-8, the transgene KGF, or the transgene AFM.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleic acid sequence of the 774 base pair human HCR (SEQ ID NO: 1). This sequence was derived from the vector pCI-CI'PX#8, deposited with the American Type Culture Collection (ATCC).

Figure 2A-C depict the transgene construct used to generate IL-8, KGF, and MCP-1 transgenic mice. Vectors are labeled as referenced in the Examples. Selected restriction enzymes are shown. "ApoE*" refers to the ApoE promoter, first exon, first intron and a portion of the second intron; "SV40PA" refers to the SV40 polyA+ sequence, as described in the Examples.

Figure 3 depicts the level of IL-8 and circulating neutrophils in both control and transgenic mice. Figure 3A shows serum IL-8 levels. Figure 3B shows circulating neutrophil levels. NT represents non-transgenic (control) mice. The numbers refer to individual lines of transgenic mice used in the analysis.

Figure 4A-C depicts a nucleic acid molecule (cDNA) of approximately 2.3 kb encoding human AFM (SEQ ID NO:23). The translated amino acid sequence of AFM is

- 9 -

also shown (SEQ ID NO:24). The amino acid positions are numbered, with -21 through -1 being the signal peptide sequence, and 1-578 being the mature protein sequence.

5

DETAILED DESCRIPTION OF THE INVENTION

Definitions

- 10 The term "operably linked" refers to the arrangement of various nucleic acid molecule elements relative to each such that the elements are functionally connected and are able to interact with each other. Such elements may include, without limitation, a
- 15 promoter, an enhancer, a polyadenylation sequence, one or more introns and/or exons, and a coding sequence of a gene of interest to be expressed (i.e., the transgene). The nucleic acid sequence elements, when properly oriented or operably linked, act together to modulate
- 20 the activity of one another, and ultimately may affect the level of expression of the transgene. By modulate is meant increasing, decreasing, or maintaining the level of activity of a particular element. The position of each element relative to other elements may be
- 25 expressed in terms of the 5' terminus and the 3' terminus of each element, and the distance between any particular elements may be referenced by the number of intervening nucleotides, or base pairs, between the elements.
- 30 The term "transgene" refers to a particular nucleic acid sequence encoding a polypeptide or a portion of a polypeptide to be expressed in a cell into which the nucleic acid sequence is inserted. The term "transgene" is meant to include (1) a nucleic acid
- 35 sequence that is not naturally found in the cell (i.e., a heterologous nucleic acid sequence); (2) a nucleic

- 10 -

acid sequence that is a mutant form of a nucleic acid sequence naturally found in the cell into which it has been inserted; (3) a nucleic acid sequence that serves to add additional copies of the same (*i.e.*, homologous) or a similar nucleic acid sequence naturally occurring in the cell into which it has been inserted; or (4) a silent naturally occurring or homologous nucleic acid sequence whose expression is induced in the cell into which it has been inserted. By "mutant form" is meant a nucleic acid sequence that contains one or more nucleotides that are different from the wild-type or naturally occurring sequence, *i.e.*, the mutant nucleic acid sequence contains one or more nucleotide substitutions, deletions, and/or insertions. In some cases, the transgene may also include a sequence encoding a leader peptide or signal sequence such that the transgene product will be secreted from the cell.

The term "promoter" refers to a nucleic acid sequence that regulates, either directly or indirectly, the transcription of a corresponding nucleic acid coding sequence to which it is operably linked. The promoter may function alone to regulate transcription, or, in some cases, may act in concert with one or more other regulatory sequences such as an enhancer or silencer to regulate transcription of the transgene.

The term "an HCR enhancer" refers to a non-coding nucleic acid sequence naturally located on human chromosome 19 within or proximal to the apoE/apoC-I gene locus, downstream of the ApoE and ApoC-I promoter/gene sequences, but upstream of the ApoC-I pseudogene sequence. As used herein, an HCR enhancer refers to any nucleic acid sequence of about 774 base pairs, and to fragment(s) thereof that has (have) biological activity. When an HCR enhancer is operably linked to both a promoter and a transgene, the HCR enhancer can (1) confer a significant degree of liver specific expression

- 11 -

of the transgene, and/or (2) can increase the level of expression of the transgene in the liver.

The term "rodent" refers to all members of the phylogenetic order *Rodentia*, such as, for example,

5 mouse, rat, hamster, squirrel, or beaver.

The term "progeny" refers to all offspring of the transgenic mammal, and includes every generation subsequent to the originally transformed transgenic mammal.

10

Preparation of the Invention

1. Preparation of DNA Constructs

15

A. Selection of Transgene

This invention contemplates expression of one or more transgenes primarily in the liver and/or the gastro-intestinal tissue of a transgenic mammal. Where
20 the transgene is expressed primarily in the liver, the gene product may be secreted into the bloodstream after synthesis. Thus, included within the scope of this invention is any transgene encoding a polypeptide to be circulated in the blood. Typically, the transgene will
25 be a nucleic acid molecule encoding a polypeptide involved in the immune response, hematopoiesis, inflammation, cell growth and proliferation, cell lineage differentiation, and/or the stress response. The transgene may be homologous or heterologous to the
30 promoter and/or to the mammal. In addition, the transgene may be a full length cDNA or genomic DNA sequence, or any fragment, subunit or mutant thereof that has at least some biological activity. Optionally, the transgene may be a hybrid nucleic acid sequence,
35 i.e., one constructed from homologous and/or heterologous cDNA and/or genomic DNA fragments. The

- 12 -

transgene may also optionally be a mutant of one or more naturally occurring cDNA and/or genomic sequences.

The transgene may be isolated and obtained in suitable quantity using one or more methods that are well known in the art. These methods and others useful for isolating a transgene are set forth, for example, in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and in Berger and Kimmel (*Methods in Enzymology: Guide to Molecular Cloning Techniques*, vol. 152, Academic Press, Inc., San Diego, CA [19AFM]).

Where the nucleic acid sequence of the transgene is known, the transgene may be synthesized, in whole or in part, using chemical synthesis methods such as those described in Engels et al. (*Angew. Chem. Int. Ed. Engl.*, 28:716-734 [1989]). These methods include, *inter alia*, the phosphotriester, phosphoramidite and H-phosphonate methods of nucleic acid synthesis.

Alternatively, the transgene may be obtained by screening an appropriate cDNA or genomic library using one or more nucleic acid probes (oligonucleotides, cDNA or genomic DNA fragments with an acceptable level of homology to the transgene to be cloned, and the like) that will hybridize selectively with the transgene DNA.

Another suitable method for obtaining a transgene is the polymerase chain reaction (PCR). However, successful use of this method requires that enough information about the nucleic acid sequence of the transgene is known so as to design suitable oligonucleotide primers useful for amplification of the appropriate nucleic acid sequence.

Where the method of choice requires the use of oligonucleotide primers or probes (e.g. PCR, cDNA or genomic library screening), the oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous so as to

- 13 -

minimize the amount of non-specific binding that will occur during library screening or PCR. The actual sequence of the probes or primers is usually based on conserved or highly homologous sequences or regions from the same or a similar gene from another organism. Optionally, the probes or primers can be degenerate.

In cases where only the amino acid sequence of the transgene is known, a probable and functional nucleic acid sequence may be inferred for the transgene using known and preferred codons for each amino acid residue. This sequence can then be chemically synthesized.

This invention contemplates the use of transgene mutant sequences. A mutant transgene is a transgene containing one or more nucleotide substitutions, deletions, and/or insertions as compared to the wild type sequence. The nucleotide substitution, deletion, and/or insertion can give rise to a gene product (*i.e.*, protein) that is different in its amino acid sequence from the wild type amino acid sequence. Preparation of such mutants is well known in the art, and is described for example in Wells *et al.* (*Gene*, 34:315 [1985]), and in Sambrook *et al.*, *supra*.

Preferred transgenes of the present invention are erythropoietin (EPO), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 9 (IL-9), interleukin 10 (IL-10), interleukin 11 (IL-11), interleukin 12 (IL-12), ENA-78 (Walz *et al.*, *J. Exp. Med.*, 174:1355-1362 [1991]; Strieter *et al.*, *Immunol. Invest.*, 21:589-596 [1992]), interferon- α , interferon- β , interferon- γ , granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor (SCF),

- 14 -

keratinocyte growth factor (KGF), monocyte
chemoattractant protein-1 (MCP-1; Furutani et al.,
supra), tumor necrosis factor (TNF), AFM, and fragments,
subunits or mutants thereof. More preferred transgenes
5 include erythropoietin, interleukin 8, MCP-1,
keratinocyte growth factor, AFM, and ENA-78. The most
preferred transgenes include human interleukin 8, human
keratinocyte growth factor, AFM, and MCP-1.

10 B. Selection of Regulatory Elements

 This invention contemplates the use of
promoters that are regulated at least in part by an HCR
enhancer which results in increased liver expression of
15 the transgene.

 The promoter may be homologous (*i.e.*, from the
same species as the mammal to be transfected with the
transgene) or heterologous (*i.e.*, from a source other
than the species of the mammal to be transfected with
20 the transgene). As such, the source of the promoter may
be any unicellular prokaryotic or eukaryotic organism,
any vertebrate or invertebrate organism, or any plant,
provided that the promoter is functional in combination
with an HCR enhancer. The more preferred promoters of
25 this invention are the ApoA-I promoter, the ApoA-II
promoter, the ApoA-IV promoter, the ApoB promoter, the
ApoC-I promoter, the ApoC-II promoter, the ApoC-III
promoter, the ApoE promoter, the albumin promoter, the
alpha feto protein promoter, the PEPCK (phosphoenol
30 pyruvate carboxykinase) promoter (EP 365,591, published
May 2, 1990), the transthyretin promoter, the SV40
promoter, the CMV promoter, and the TK (thymidine
kinase) promoter. The most preferred promoters of this
group are ApoE, ApoC-I, and ApoA-IV. The most preferred
35 promoters are human ApoE and human ApoC-I.

- 15 -

The preferred HCR enhancer element contemplated herein is a non-coding DNA sequence located on human chromosome 19 within, or proximal to, the apoE/apoC-I gene locus, downstream of the ApoE and ApoC-I genes, but upstream of the ApoC-I pseudogene. The approximately 774 base pair HCR has been deposited under the Budapest Treaty with the American Type Culture Collection (ATCC; 12301 Parklawn Drive, Rockville, MD 20852) as accession number 69422. The date of deposit is September 17, 1993. Fragments of this HCR sequence are also contemplated herein, provided that the fragment has the property of modulating expression of a transgene in the liver (*i.e.*, is biologically active).

The promoter sequences of this invention may be obtained by any of several methods well known in the art. Typically, promoters useful herein will have been previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the promoter may have been sequenced. For those promoters whose DNA sequence is known, the promoter may be synthesized using the methods described above for transgene synthesis.

Where all or only portions of the promoter sequence are known, the promoter may be obtained using PCR and/or by screening a genomic library with suitable oligonucleotide and/or promoter sequence fragments from the same or another species.

Where the promoter sequence is not known, a fragment of DNA containing the promoter may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion using one or more carefully selected enzymes to isolate the proper DNA fragment. After digestion, the desired fragment is isolated by

- 16 -

agarose gel purification, Qiagen column or other methods known to the skilled artisan. Selection of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

5

C. Selection of Other Vector Components

In addition to the transgene, the promoter, and the HCR enhancer, the vectors useful in this invention typically contain one or more other elements useful for (1) optimal functioning of the vector in the mammal into which the vector is transfected, and (2) amplification of the vector in bacterial or mammalian host cells. Each of these elements will be positioned appropriately in the vector with respect to each other element so as to maximize their respective activities. Such positioning is well known to the ordinary skilled artisan. The following elements may be optionally included in the vector as appropriate.

20

i. Signal Sequence Element

For those embodiments of the invention where the transgene is to be secreted, a signal sequence, is frequently present to direct the polypeptide encoded by the transgene out of the cell where it is synthesized. Typically, the signal sequence is positioned in the coding region of the transgene towards or at the 5' end of the coding region. Many signal sequences have been identified, and any of them that are functional in the transgenic tissue may be used in conjunction with the transgene. Therefore, the signal sequence may be homologous or heterologous to the transgene, and may be homologous or heterologous to the transgenic mammal. Additionally, the signal sequence may be chemically

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- 17 -

synthesized using methods set forth above. However, for purposes herein, preferred signal sequences are those that occur naturally with the transgene (i.e., are homologous to the transgene).

5

ii. Membrane Anchoring Domain Element

In some cases, it may be desirable to have a transgene expressed on the surface of a particular intracellular membrane or on the plasma membrane. Naturally occurring membrane proteins contain, as part of the translated polypeptide, a stretch of amino acids that serve to anchor the protein to the membrane. However, for proteins that are not naturally found on the membrane, such a stretch of amino acids may be added to confer this feature. Frequently, the anchor domain will be an internal portion of the protein and thus will be engineered internally into the transgene. However, in other cases, the anchor region may be attached to the 5' or 3' end of the transgene. Here, the anchor domain may first be placed into the vector in the appropriate position as a separate component from the transgene. As for the signal sequence, the anchor domain may be from any source and thus may be homologous or heterologous with respect to both the transgene and the transgenic mammal. Alternatively, the anchor domain may be chemically synthesized using methods set forth above.

30

iii. Origin of Replication Element

This component is typically a part of prokaryotic expression vectors purchased commercially, and aids in the amplification of the vector in a host cell. If the vector of choice does not contain an origin of replication site, one may be chemically

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- 18 -

synthesized based on a known sequence, and ligated into the vector.

5

iv. Transcription Termination Element

This element is typically located 3' to the
10 transgene coding sequence and serves to terminate
transcription of the transgene. Usually, the
transcription termination element is a polyadenylation
signal sequence. While the element is easily cloned
from a library or even purchased commercially as part of
15 a vector, it can also be readily synthesized using
methods for nucleic acid synthesis such as those
described above.

v. Intron Element

20

In many cases, transcription of the transgene
is increased by the presence of one or more introns on
the vector. The intron may be naturally occurring
within the transgene sequence, especially where the
25 transgene is a full length or a fragment of a genomic
DNA sequence. Where the intron is not naturally
occurring within the DNA sequence (as for most cDNAs),
the intron(s) may be obtained from another source. The
intron may be homologous or heterologous to the
30 transgene and/or to the transgenic mammal. The position
of the intron with respect to the promoter and the
transgene is important, as the intron must be
transcribed to be effective. As such, where the
transgene is a cDNA sequence, the preferred position for
35 the intron is 3' to the transcription start site, and 5'
to the polyA transcription termination sequence.

- 19 -

Preferably for cDNA transgenes, the intron will be located on one side or the other (i.e., 5' or 3') of the transgene sequence such that it does not interrupt the transgene sequence. Any intron from any source, including any viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell(s) into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector. A preferred intron is intron 1 of the human ApoE gene.

vi. Selectable Marker(s) Element

Selectable marker genes encode proteins necessary for the survival and growth of transfected cells grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanomycin for prokaryotic host cells, and neomycin, hygromycin, or methotrexate for mammalian cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for cultures of *Bacilli*.

All of the elements set forth above, as well as others useful in this invention, are well known to the skilled artisan and are described, for example, in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and Berger et al., eds. (*Guide to Molecular Cloning Techniques*, Academic Press, Inc., San Diego, CA [19AFM]).

- 20 -

D. Construction of Vectors

The vectors most useful in practicing this invention are those that are compatible with prokaryotic cell hosts. However, eukaryotic cell hosts, and vectors compatible with these cells, are within the scope of the invention.

In certain cases, some of the various vector elements may be already present in commercially available vectors such as pUC18, pUC19, pBR322, the pGEM vectors (Promega Corp, Madison, WI), the pBluescript® vectors such as pBIISK+/- (Stratagene Corp., La Jolla, CA), and the like, all of which are suitable for prokaryotic cell hosts.

However, where one or more of the elements are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are comparable to the methods set forth above for obtaining a transgene (i.e., synthesis of the DNA, library screening, and the like).

Preferred vectors of this invention are the pGEM and the pBluescript® vectors. The most preferred vector is pBIISK+.

Vectors used for amplification of the transgene and/or for transfection of the mammalian embryos are constructed using methods well known in the art. Such methods include, for example, the standard techniques of restriction endonuclease digestion, ligation, agarose and acrylamide gel purification of DNA and/or RNA, column chromatography purification of DNA and/or RNA, phenol/chloroform extraction of DNA, DNA sequencing, polymerase chain reaction amplification, and the like, as set forth in Sambrook et al., *supra*.

The final vector used to practice this invention is typically constructed from a starting

- 21 -

vector such as a commercially available vector. This vector may or may not contain some of the elements to be included in the completed vector. If none of the desired elements are present in the starting vector, each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to "blunt" the ends to be ligated together in order to obtain a satisfactory ligation. Blunting is accomplished by first filling in "sticky ends" using Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook et al., *supra*.

Alternatively, two or more of the elements to be inserted into the vector may first be ligated together (if they are to be positioned adjacent to each other) and then ligated into the vector.

One other method for constructing the vector to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors will be generated due to improper ligation or insertion of the elements, however the functional vector may be identified and selected by restriction endonuclease digestion.

After the vector has been constructed, it may be transfected into a prokaryotic host cell for amplification. Cells typically used for amplification are *E coli* DH5-alpha (Gibco/BRL, Grand Island, NY) and other *E. coli* strains with characteristics similar to DH5-alpha.

Where mammalian host cells are used, cell lines such as Chinese hamster ovary (CHO cells; Urlab et al., *Proc. Natl. Acad. Sci USA*, 77:4216 [1980])) and

- 22 -

human embryonic kidney cell line 293 (Graham et al., *J. Gen. Virol.*, 36:59 [1977]), as well as other lines, are suitable.

Transfection of the vector into the selected
5 host cell line accomplished using such methods as calcium phosphate, electroporation, microinjection, lipofection or DEAE-dextran method. The method selected will in part be a function of the type of host cell to be transfected. These methods and other suitable
10 methods are well known to the skilled artisan, and are set forth in Sambrook et al., *supra*.

After culturing the cells long enough for the vector to be sufficiently amplified (usually overnight for *E. coli* cells), the vector (often termed plasmid at
15 this stage) is isolated from the cells and purified. Typically, the cells are lysed and the plasmid is extracted from other cell contents. Methods suitable for plasmid purification include *inter alia*, the alkaline lysis mini-prep method (Sambrook et al.,
20 *supra*).

E. Preparation of Plasmid For Insertion into the Embryo

Typically, the plasmid containing the
25 transgene is linearized using a selected restriction endonuclease prior to insertion into the embryo. In some cases, it may be preferable to isolate the transgene, promoter, and regulatory elements as a linear fragment from the other portions of the vector, thereby
30 injecting only a linear nucleic acid sequence containing the transgene, promoter, intron (if one is to be used), enhancer, polyA sequence, and optionally a signal sequence or membrane anchoring domain into the embryo. This may be accomplished by cutting the plasmid so as to
35 remove the nucleic acid sequence containing these

- 23 -

elements, and purifying this region using agarose gel electrophoresis or other suitable purification methods.

5

2. Production of Transgenic Mammals

Transgenic mammals may be prepared using
10 methods well known to the skilled artisan. For example, to prepare transgenic rodents such as mice, methods such as those set forth by Hogan et al., eds. (*Manipulating The Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
15 [1986]) may be employed.

The specific line(s) of any mammalian species used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryos, and good reproductive
20 fitness. For example, when transgenic mice are to be produced, lines such as C57/BL6 x DBA2 F1 cross, or FVB lines are often used (obtained commercially from Charles River Labs, Boston, MA). The line(s) used to practice this invention may themselves be transgenics, and/or may
25 be knockouts (i.e., mammals which have one or more genes partially or completely suppressed).

The age of the mammals that are used to obtain embryos and to serve as surrogate hosts is a function of the species used, but is readily determined by one of
30 ordinary skill in the art. For example, when mice are used, pre-puberal females are preferred, as they yield more embryos and respond better to hormone injections.

Similarly, the male mammal to be used as a stud will normally be selected by age of sexual
35 maturity, among other criteria.

- 24 -

Administration of hormones or other chemical compounds may be necessary to prepare the female for egg production, mating, and/or reimplantation of embryos. The type of hormones/cofactors and the quantity used, as well as the timing of administration of the hormones will vary for each species of mammal. Such considerations will be readily apparent to one of ordinary skill in the art

Typically, a primed female (i.e., one that is producing eggs that can be fertilized) is mated with a stud male, and the resulting fertilized embryos are then removed for introduction of the transgene(s). Alternatively, eggs and sperm may be obtained from suitable females and males and used for *in vitro* fertilization to produce an embryo suitable for introduction of the transgene.

Normally, fertilized embryos are incubated in suitable media until the pronuclei appear. At about this time, exogenous nucleic acid comprising the transgene of interest is introduced into the female or male pronucleus. In some species such as mice, the male pronucleus is preferred.

Introduction of nucleic acid may be accomplished by any means known in the art such as, for example, microinjection, electroporation, or lipofection. Following introduction of the transgene nucleic acid sequence into the embryo, the embryo may be incubated *in vitro* for varying amounts of time, or reimplanted into the surrogate host, or both. *In vitro* incubation to maturity is within the scope of this invention. One common method is to incubate the embryos *in vitro* for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

Reimplantation is accomplished using standard methods. Usually, the surrogate host is anesthetized, and the embryos are inserted into the oviduct. The

- 25 -

number of embryos implanted into a particular host will vary by species, but will usually be comparable to the number of offspring the species naturally produces.

Transgenic offspring of the surrogate host may
5 be screened for the presence and/or expression of the transgene by any suitable method. Screening is often accomplished by Southern blot or Northern blot analysis, using a probe that is complementary to at least a portion of the transgene. Western blot analysis using
10 an antibody against the protein encoded by the transgene may be employed as an alternative or additional method for screening for the presence of the transgene product. Typically, DNA is prepared from tail tissue (about 1 cm is removed from the tip of the tail) and analyzed by
15 Southern analysis or PCR for the transgene. Alternatively, the tissues or cells believed to express the transgene at the highest levels are tested for the presence and expression of the transgene using Southern analysis or PCR, although any tissues or cell types may
20 be used for this analysis.

Alternative or additional methods for evaluating the presence of the transgene include, without limitation, suitable biochemical assays such as enzyme and/or immunological assays, histological stains
25 for particular markers or enzyme activities, and the like. Analysis of the blood may also be useful to detect the presence of the transgene product in the blood, as well as to evaluate the effect of the transgene on the levels of various types of blood cells
30 and other blood constituents.

Progeny of the transgenic mammals may be obtained by mating the transgenic mammal with a suitable partner, or by *in vitro* fertilization of eggs and/or sperm obtained from the transgenic mammal. Where mating
35 with a partner is to be performed, the partner may or may not be transgenic and/or a knockout; where it is

- 26 -

transgenic, it may contain the same or a different transgene, or both. Alternatively, the partner may be a parental line. Where *in vitro* fertilization is used, the fertilized embryo may be implanted into a surrogate
5 host or incubated *in vitro*, or both. Using either method, the progeny may be evaluated for the presence of the transgene using methods described above, or other appropriate methods.

The transgenic mammals of this invention may
10 be used to generate one or more cell lines. Such cell lines have many uses, as for example, to evaluate the effect(s) of the transgene on a particular tissue or organ, and to screen compounds that may affect the level of activity of the transgene in the tissue. Such
15 compounds may be useful as therapeutics to modulate the activity of the transgene.

Production of cell lines may be accomplished using a variety of methods, known to the skilled artisan. The actual culturing conditions will depend on
20 the tissue and type of cells to be cultured. Various media containing different concentrations of macro and micro nutrients, growth factors, serum, and the like, can be tested on the cells without undue experimentation to determine the optimal conditions for growth and
25 proliferation of the cells. Similarly, other culturing conditions such as cell density, media temperature, and carbon dioxide concentrations in the incubator can also readily be evaluated.

The transformed mammals, their progeny, and
30 transgenic cell lines of the present invention provide several important uses that will be readily apparent to one of ordinary skill in the art. The mammals and cell lines are particularly useful for (a) providing and evaluating the potential of treatments (such as gene
35 therapy) for a variety of conditions and diseases, and/or (b) screening compounds that have potential as

- 27 -

prophylactics or therapeutics. Such uses may be found for (1) conditions caused by inflammation, (2) immune system disorders, (3) epithelial cell repair (skin, lung and/or intestinal epithelia), (4) hematopoiesis, and/or

5 (5) disorders caused by various physical and/or mental stresses. For example, transgenic mammals or cell lines containing the transgene for IL-8 will be useful for identifying compounds that modulate neutrophil migration; transgenic mammals containing the transgene

10 KGF will be useful for evaluating epithelial tissue repair, and identifying compounds that affect this process.

In the case of transgenic mammals, screening of candidate compounds is conducted by administering the

15 compound(s) to be tested to the mammal, over a range of doses, and evaluating the mammal's physiological response to the compound(s) over time. Administration may be by any appropriate means such as, for example, oral administration, or administration by injection,

20 implantation, or transdermal delivery, depending on the chemical nature of the compound being evaluated. In some cases, it may be appropriate to administer the compound in conjunction with other compounds or co-factors that might enhance the efficacy of the compound.

25 In screening cell lines for compounds useful in treating the above mentioned problems, the compound is added to the cell culture medium at the appropriate time, and the cellular response to the compound is evaluated over time using the appropriate biochemical

30 and/or histological assays. In some cases, it may be appropriate to apply the compound of interest to the culture medium in conjunction with other compounds or co-factors that might enhance the efficacy of the compound.

35

- 28 -

The invention will be more fully understood by reference to the following examples. They should not be construed in any way as limiting the scope of the present invention.

5

EXAMPLES

10 Example 1: Preparation of a HCR-IL-8 Transgenic MouseA. Construction of Transgene and Vectors

15 A diagram depicting the overall cloning strategy used herein is set forth in Figure 2.

A *PstI*-*XbaI* DNA fragment of about 774 base pairs (containing HCR sequence) obtained from the human apoC-I/C-I' intergenic region on chromosome 19 (Simonet et al. [1993], *supra*) was subcloned into the *PstI*-*XbaI* sites of pUC19 (New England Biolabs, Beverly, MA). The
20 resulting plasmid was designated pCI-CI'PX#8. This plasmid has been deposited on September 17, 1993 with the ATCC as accession number 69422.

An approximately 1.45 kb *Kpn-I* fragment containing
25 a contiguous piece of DNA consisting of 650 bp of the human ApoE gene 5'-flanking sequence, the first exon, first intron and a portion of the second exon of the ApoE gene was excised from the vector pHE54 (Simonet et al., [1993], *supra*). This fragment of about 1.45 kb was
30 inserted by ligation into the *Kpn-I* cloning site of pCI-CI'PX#8. After ligation, the plasmid was transfected into *E coli* strain DH5-alpha (Gibco/BRL, Grand Island, NY). The cells were plated out on standard LB (Luria broth) or TB (Terrific broth) plus ampicillin medium
35 (Sambrook et al., *supra*) on agarose plates, and grown up overnight at 37°C.

- 29 -

Colonies were then selected and grown up overnight in standard LB medium in the presence of ampicillin for amplification. After amplification, plasmid DNA from each amplified colony was prepared using the standard alkaline lysis miniprep method (Sambrook et al., *supra*), and the plasmid DNA was purified using a Qiagen column (Qiagen Corp., Chatsworth, CA). Purified plasmid was then digested with the restriction endonuclease BamHI and analyzed by agarose gel electrophoresis. Of 18 colonies analyzed, 6 were found to have a single insert ligated in the desired orientation. The resulting construct containing the HCR upstream of the ApoE promoter and exon/intron sequence was designated pHCR-HEP.

The approximately 2.2 kb HCR enhancer-promoter-intron cassette was excised from pHCR-HEP as either a *PstI-EcoRI* fragment or a *HindIII-EcoRI* fragment. Each of these fragments were ligated into pBIISK+ (Stratagene Corp., La Jolla, CA) to generate the plasmids pHCR-HEP BS (PE) (*PstI-EcoRI* fragment) and pHCR-HEP BS (HE) (*HindIII-EcoRI* fragment).

The eukaryotic expression vector V19-10 was used as a template for amplification of the SV40 polyA+ signal. This vector was constructed by inserting a 592 base pair *AatII/ClaI* fragment containing the origin of replication sequence from bacteriophage M13 into the eukaryotic expression vector V19-8 (described in WO 91/05795, published May 2, 1991). The 242 base pair polyA+ sequence from V19-10 was amplified as a *NotI-SacII* fragment or a *HindIII-XhoI* fragment using PCR. The primers used for PCR amplification were:

NotI-SacII fragment:

Primer 1: CTCTAGAAAGCTTAATTCAGTC (SEQ ID NO: 2)

- 30 -

Primer 2: TCCCCGCGGGGAAGAGCGCAGAGCTCGG (SEQ ID NO: 3)

Thirty cycles of amplification were conducted as follows: Denaturation was at 94°C for 30 seconds; annealing was at 56°C for 30 seconds; and extension was at 72°C for 30 seconds.

HindIII-XhoI fragment:

10 Primer 3: CTCTAGAAAGCTTAATTCAGTC (SEQ ID NO: 4)

Primer 4: CTGGATCTCGAGGTACCCGGGGATCATAATC (SEQ ID NO: 5)

Thirty cycles of amplification were conducted as follows: Denaturation was at 94°C for 30 seconds; annealing was at 57°C for 30 seconds; and extension was at 72°C for 30 seconds.

The PCR fragments were sequenced and showed 100% homology to the template. The fragments were then subcloned into *NotI-SacII* cut or *HindIII-XhoI* cut pBIISK+, to generate the plasmids pBS-PA (NS) and pBS-PA (HX), respectively.

The human IL-8 cDNA was obtained by screening a human peripheral blood lymphocyte cDNA library, prepared as follows:

Peripheral blood lymphocytes were isolated from freshly prepared buffy coats, on a ficol-paque step gradient (Pharmacia, Uppsala, Sweden). Mononuclear cells present in the interphase of the gradient were removed and washed with PBS three times. The cells were then suspended in the medium RPMI 1640 + 10% FCS (fetal calf serum). About 5 million cells/ml were incubated with pokeweed mitogen (10 ug/ml, Sigma Chemical Corp., St. Louis, MO) for 19 hours, followed by addition of cycloheximide to a final concentration of 10 ug/ml for

- 31 -

an additional 6 hours. Incubation was carried out at 37°C and 5% CO₂.

Total RNA was isolated from activated lymphocytes using the guanidium thiocyanate-CsCl technique (Chirgwin et al., *Biochem.*, 18: 5294-5299 [1979]). Polyadenylated RNA was selected by oligo(dT) chromatography. The polyA⁺ RNA was then ethanol precipitated and centrifuged. The final pellet was dissolved in water and kept in liquid nitrogen in aliquots.

About 5 ug of polyA⁺ RNA were used for cDNA library construction. After denaturation with methyl mercury hydroxide, oligo(dT)-primed double strand cDNA was synthesized following the procedure set forth in Sambrook et al., *supra*, followed by methylation with *EcoRI* and Alu methylases. The technique of Dorssers et al., (*Nuc. Acid. Res.*, 15: 3629, [19AFM]) was used to introduce *EcoRI* and *HindIII* sites on the 5' and 3' ends of the cDNAs, respectively. After digestion with *EcoRI* and *HindIII* restriction enzymes, cDNAs that were larger than 500 base pairs were isolated from an agarose gel by electroelution. The eukaryotic expression vector V19-10 (described above), was digested with *EcoRI* and *HindIII* and was then ligated with the cDNAs. These new plasmids containing cDNA inserts were transfected into competent DH5 alpha cells (GIBCO-BRL, Gaithersburg, MD). The cDNA library was frozen in aliquots at -80°C after addition of DMSO to 7% (Okayama & Berg, *Mol. Cell. Biol.*, 2: 161-170, 1982).

A mixed oligonucleotide probe was designed on the basis of similarity in nucleotide sequences surrounding and coding for the signal peptidase cleavage site of a number of cytokines. The sequence of this degenerate probe was:

ATGTCGACMWC SVTGCMCCHRYMYSMYCYA (SEQ ID NO: 6)

- 32 -

In this sequence, M, W, S, V, R, Y, and H represent degenerate nucleotides. M represents A or C; W represents A or T; S represents C or G; V represents A or C or G; R represents A or G; Y represents C or T; and
5 H represents A or C or T.

Using this probe, a cDNA encoding IL-8 was obtained and sequenced for homology comparison to the published sequence for IL-8 (Furutani et al., Biophys. Biochem.
10 Res. Comm., 159:249-255 [1989]). The IL-8 cDNA clone was then used as a template to PCR amplify a *SpeI*-*NotI* fragment of the cDNA. Amplification was accomplished using the following oligonucleotide primers:

15 Primer 5: GGACTAGTCCAGAGCACACAAGCTTCTAG (SEQ ID NO: 7)

Primer 6: ATAAGAATGCGGCCGCTAAACTATTGCATCTGGCAACCC (SEQ ID NO: 8)

Thirty cycles of amplification were conducted as
20 follows: Denaturation was at 94°C for 30 seconds; annealing was at 54°C for 30 seconds; and extension was at 72°C for 30 seconds.

The amplified fragment was then subcloned into *SpeI*-*NotI* cut pIIBS-PA (NS) to produce the plasmid pIL-8
25 PA. The amplified IL-8 sequence, which lacked a portion of the 3' untranslated sequence of the original IL-8 cDNA, was sequence verified and found to be 100% homologous to human IL-8 in the coding region.

The polyadenylated IL-8 cDNA was put under the
30 control of the HCR enhancer and the ApoE promoter by excising the HCR-ApoE promoter-intron cassette from the vector pHCR-HEP BS(HE) as a *XhoI*-*SpeI* fragment. This fragment was then subcloned into *XhoI*-*SpeI* cut pIL-8 PA to generate the plasmid pHCR-HEP IL-8 PA (abbreviated
35 HE8).

- 33 -

For microinjection, the plasmid HE8 was digested with restriction enzymes *XhoI*, *ScaI* and *AflIII*, and the approximately 3.3 Kb *XhoI*-*AflIII* insert fragment containing the HCR, the ApoE promoter, the ApoE first
5 exon, first intron, a portion of the second exon, the human IL-8 cDNA and the SV40 poly-adenylation signal was purified on a 0.8% ultrapure DNA agarose gel (BRL Corp., Bethesda, MD) and diluted to 1 ng/ul in 5mM Tris, pH 7.4, 0.2mM EDTA. About 2 to 3 picoliters of this
10 solution were injected into the male pronucleus of each mouse embryo.

To prepare a liver expression vector to make transgenic mammals containing the transgenes KGF or MCP-1, the approximately 242 base pair *HindIII*-*XhoI* insert
15 fragment from pBS PA (HX) was isolated and subcloned into *HindIII*-*XhoI* cut pHCR-HEP BS (PE). The resulting vector, pliv(BSK), has a polylinker region containing *EcoRI*, *EcoRV*, and *HindIII* restriction sites downstream of the HCR-ApoE promoter-intron cassette and upstream of
20 the SV40 poly-adenylation signal.

B. Preparation of Embryos and Microinjection

Pregnant mare's serum ("PMS"), supplying
25 Follicle Stimulating Hormone ("FSH") was administered to female mice of the strain BDF1 (Charles River Labs, Boston, MA) about three days prior to the day of microinjection. PMS (obtained from Sigma Chemicals) was prepared as a 50 I.U./ml solution in Phosphate Buffered
30 Saline and injected intraperitoneally at 0.1 ml (5 I.U.) per animal. Human Chorionic Gonadotropin ("HCG"), supplying Luteinizing Hormone ("LH") was administered 45-48 hours after the PMS injections. HCG was also prepared as a 50 I.U./ml solution in PBS and injected IP
35 (intraperitoneally) at 0.1 ml per animal. Females were placed with stud males of the same strain immediately

- 34 -

after HCG injections. After mating, the females were examined for a vaginal copulation plug. The appearance of an opaque white plug indicated a successful mating.

Successfully mated females were sacrificed by cervical dislocation, and both oviducts were rapidly removed and placed in M2 medium (Hogan et al., eds., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, pp 249-257 [1986]). The oviducts were transferred individually from M2 medium to PBS containing 300 µg/ml hyaluronidase (Sigma Corp., St. Louis, MO.) in a round bottom dissection slide. The embryos were teased out of the oviduct and allowed to settle at the bottom of the slide as the cumulus cells detached from the embryos. When the cumulus masses were disaggregated (about 5 minutes) the embryos were transferred through two washes of M2 medium and the fertilized embryos were separated from unfertilized and abnormal embryos. The fertilized embryos were then transferred through 5% CO₂ equilibrated M16 medium (Hogan et al., *supra*), placed in equilibrated microdrop dishes containing M16 medium under paraffin oil and returned to the incubator.

Fertilized single-cell embryos from BDF1 xBDF1-bred mice were selected in M16 medium and incubated about 5 hours at 37°C until the pronuclei appeared. Embryos were then transferred into M2 medium in a shallow depression slide under paraffin oil and placed under the microscope. The pronuclei were easily visible under 200X magnification. Using suction on the holding pipet, a single embryo was selected and rotated such that the male pronucleus was away from the holding pipet. Approximately 2 to 3 picoliters of solution containing the DNA construct at about 1 microgram per ml was injected into one of the pronuclei, preferably the male pronucleus. Following the injection, the embryos

- 35 -

were returned to incubation for 18 hours and reimplanted the next day into foster pseudopregnant females.

Reimplantations were performed on anesthetized female mice of strain CD1 using a dissecting microscope.

- 5 A pseudo-pregnant female mouse was anaesthetized with 0.017-0.020 ml/g body weight of avertin, injected IP. The mouse was placed under the dissecting microscope and the incision area was disinfected with 70% ethanol. The ovary was exteriorized and the bursal sac that surrounds
10 the ovary and the oviduct was carefully pulled open. The ovary and oviduct were separated to expose the opening of the oviduct (termed the infundibulum). Surviving embryos were then removed from the incubator and loaded into the reimplantation pipet. The tip of
15 the pipet was inserted several millimeters into the infundibulum and gentle pressure was used to deliver the embryos into the oviduct. About 10 to 20 2-cell embryos were implanted per mouse, resulting in a litter size of about 3 to 12. The ovary then was returned to the
20 peritoneum, and the body wall and then the skin were sutured.

C. Identification of Transgenic Mice

- 25 Of 52 mice born after embryo injections, 9 contained the IL-8 transgene as assayed by PCR amplification. About 1 cm of the tail of each mouse was removed, and DNA was prepared using the technique set forth by Hogan et al., supra. The DNA was then
30 subjected to PCR analysis using the following primers:

Primer 7: GCCTCTAGAAAGAGCTGGGAC (SEQ ID NO: 9)

Primer 8: CGCCGTGTTCCATTATGAGC (SEQ ID NO: 10)

- 36 -

The PCR amplification procedure was denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. Thirty cycles were performed.

5 The resultant transgenic mice harboring the transgene in their genome are termed the founder mice. The founder mice were backcrossed to strain BDF1 mice to generate heterozygous F1 transgenic mice.

10 To evaluate the F1 transgenic mice for the presence and effect of IL-8, blood was obtained and analyzed as follows.

15 Quantitation of serum IL-8 levels were determined using an Elisa kit for human IL-8 (obtained from Biosource International, Camarillo, CA) and following the manufacturer's protocol. The results are shown in Figure 3A. As can be seen, three of the lines of F1 transgenic mice (HE8 lines 7, 26, and 51) had levels of about 100 ng/ml or higher, while no IL-8 was detected in the serum of the non-transgenic (NT) mice.

20 Circulating white blood cells in the serum of the F1 transgenic and non-transgenic mice were counted using a Sysmex F-800 blood cell counter (Toa Medical Electronics Co., LTD, Kobe, Japan) and following the manufacturer's protocol. Prior to counting, red blood
25 cells were lysed with Quicklyser™ (Toa Medical Electronics Co., LTD, Kobe, Japan), following the manufacturer's protocol. For differential leukocyte analysis, about 3 µl of whole blood were spread on a glass slide and subjected to Wright's-Giemsa staining.
30 At least 100 cells were counted from each slide by visualizing the cells under a 100x oil emersion lens on an Olympus CH2 student microscope. Neutrophils were distinguished from lymphocytes, macrophages, eosinophils, and basophils by their multinucleated
35 structures. For all lines reported, at least five individual F1 heterozygotes were bled and analyzed.

- 37 -

Absolute neutrophil levels were determined by multiplying the percentage of neutrophils on the Wright's-Giemsa stained slides by the total white blood cell count obtained from the Sysmex counter. The

- 5 results are shown in Figure 3B. Three of the F1 transgenic lines evaluated (HE8 lines 7, 26, and 51) had a circulating neutrophil level of greater than 6,000/ μ l blood, while the non-transgenic (NT) mice had a level of under 1,000/ μ l blood.

10

Example 2: Preparation of a HCR-KGF Transgenic Mouse

- The gene encoding human KGF (keratinocyte growth factor) was obtained by PCR amplification of the gene
15 from a normal human dermal fibroblast cDNA library. PCR amplification of KGF was accomplished using the following two oligonucleotide primers:

Primer 9: CAATCTACAATTACAGA (SEQ ID NO: 11)

20

Primer 10: TTAAGTTATTGCCATAGG (SEQ ID NO: 12)

- The conditions for PCR were: denaturation at 92°C for 20 seconds; anneal at 55-40°C for 20 seconds (this
25 consisted of 2 cycles at 55°C, followed by 2 cycles at 45°C, which was followed by 28 cycles at 40°C); and extension at 72°C for 30 seconds. Thirty cycles total were performed.

- To introduce *HindIII* and *BglIII* restriction sites to
30 the ends of the KGF cDNA, the cDNA was PCR amplified using the following two oligonucleotide primers:

Primer 11: AACAAAGCTTCTACAATTACAGATAGGA (SEQ ID NO: 13)

- 35 Primer 12: AACAGATCTTAAGTTATTGCCATAGG (SEQ ID NO: 14)

- 38 -

The conditions for PCR were: denaturation at 92°C for 20 seconds; anneal at 45°C for 20 seconds; and elongation at 72°C for 30 seconds. Thirty cycles were performed.

5 After amplification, the KGF cDNA was purified and digested with *HindIII* and *BglIII*, and then ligated into the vector pCFM3006. This vector was prepared from the vector pCFM836 (described in U.S. Patent No. 4,710,473, issued December 1, 1994). The two endogenous *NdeI*
 10 restriction sites in pCFM836 were removed by cutting pCFM836 with *NdeI*, filling in the cut ends of the vector using T4 polymerase, and then re-ligating the vector by blunt end ligation. Next, the DNA sequence between the *AatII* and *KpnI* sites of the now modified pCFM836 was
 15 altered using the technique of PCR overlapping oligonucleotide mutagenesis. The following changes at the base pair positions listed were made (the base pair position changes are relative to the *BglIII* site on pCFM836 which is position #180):

20	<u>plasmid bp #</u>	<u>bp changed</u>
	# 428	G/C
	# 509	A/T
	# 617	insert two G/C bp
25	# 978	C/G
	# 992	A/T
	# 1002	C/G
	# 1005	T/A
	# 1026	T/A
30	# 1045	T/A
	# 1176	T/A
	# 1464	T/A
	# 2026	bp deletion
	# 2186	T/A
35	# 2479	T/A
	# 2498-2501	<u>GTCA</u>

- 39 -

# 2641-2647	bp deletion
# 3441	A/T
# 3649	T/A

5 The KGF cDNA in this vector was used as a template for amplification. A 710 base pair *HindIII* fragment of KGF was amplified using PCR and the following two oligonucleotide primers:

10 Primer 13: CGATCGTAAGCTTGGTCAATGACCTAGGAGTAAC (SEQ ID NO: 15)

Primer 14: CGATCGTAAGCTTGCGGATCCTAAGTTATTGCC (SEQ ID NO: 16)

Amplification was conducted for 30 cycles. Denaturation
15 was at 94°C for 30 seconds, annealing was at 58°C for 20 seconds, and elongation was at 72°C for 30 seconds. The amplified fragment was purified by agarose gel electrophoresis and then ligated into the vector plivBsk (described in Example 1; shown in Figure 2). *E. coli*
20 cells were then transformed with the ligation mixture and plated out for overnight incubation. After incubation, colonies were selected, grown up, and the plasmids analyzed for those containing KGF in the proper orientation. The orientation of the plasmid KGF was
25 determined by restriction endonuclease digestion with *EcoRI*. Clones with the proper orientation were grown up and the plasmid purified using a Qiagen column (Qiagen Corp., Chatsworth, CA). Several clones were sequenced to verify the orientation and sequence of the KGF.

30 DNA to be used in microinjection of the embryos was prepared by cutting the vector containing KGF with *SpeI* and *XhoI* to obtain a DNA fragment containing (in order) the HCR, ApoE promoter, KGF, and polyA sequences. This DNA was gel purified and prepared as described in
35 Example 1. Microinjection and implantation into pseudopregnant mice were as described in Example 1.

- 40 -

Example 3: Preparation of a HCR-MCP-1 Transgenic Mouse

The cDNA encoding human MCP-1 was obtained by
5 screening the human peripheral blood lymphocyte library
described in Example 1 with the following probe:

CTGTSYCTSCTSNSTMTWGTWGCYGSCT (SEQ ID NO: 17)

10 In the probe sequence, S represents C or G; Y represents
T or C; N represents A or T or C or G; M represents C or
A; and W represents A or T.

A clone of about 850 base pairs was obtained
using this probe and was inserted into the vector V19-8
15 (described in Example 1). This clone was then sequenced
for identification, and found to be homologous to the
published sequence for MCP-1 (Matsushima et al., *J. Exp.
Med.*, 167:1883-1893 [1988]).

The MCP-1 cDNA was excised from the vector
20 V19-8 as an approximately 350 base pair *EcoRI*-*PstI*
fragment, and was ligated into the vector pUC19
previously cut with *EcoRI* and *HindIII*. The cDNA was
then removed as an *EcoRI*-*HindIII* fragment and inserted
into the vector plivBSK. The vector containing the cDNA
25 was called HEMF. This vector was transformed into
E. coli strain DH5 alpha for amplification. After
culturing the cells overnight, the plasmid was isolated
and purified using the alkaline lysis method, followed
by cesium chloride centrifugation.

30 After centrifugation, the plasmid was digested
with the restriction enzymes *SpeI*, *XhoI*, and *ScaI*, and
the approximately 2.8 kilobase DNA fragment containing
the HCR, ApoE promoter and first intron, MCP-1 cDNA, and
the SV40 polyA sequence was isolated. This DNA was gel
35 purified and prepared for microinjection as described in
Example 1. Microinjection of embryos and implantation

- 41 -

of embryos into pseudopregnant mice were as described in Example 1.

Of 130 offspring analyzed, 5 contained the MCP-1 transgene as detected using PCR analysis.

5

Example 4: Preparation of a HCR-AFM Transgenic Mouse

The cDNA encoding human AFM was obtained as follows:

The polymerase chain reaction (PCR) was used to amplify a portion of the cDNA encoding AFM. PCR was first performed in a total volume of about 100 μ l using approximately one nanogram of Quick Clone human liver cDNA (Clontech, cat. no. 7113-1) as the template and standard PCR buffer (Perkin-Elmer Cetus). About 1 μ M of each of the following two degenerate primers was also used in this PCR reaction.

ACGCTGAATTCGCCARAARTTYATHGARGAYAA (SEQ ID NO:18)

ACGCTAAGCTTGCRTCYTTTADATYTGACDAT (SEQ ID NO:19)

25

In these primer sequences, R represents A or G; Y represents T or C; N represents A or T or C or G; D represents G or A or T; H represents A or C or T.

The conditions used for the PCR reaction were as follows: 95°C for 8 min (1 cycle); 94°C for 1 min, 34°C for 10 min and 72°C for 2 min (3 cycles); 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min (45 cycles); 72°C for 5 min (1 cycle).

An approximately 1 μ l aliquot of amplified DNA obtained from this PCR was used as a template for a

35

- 42 -

second PCR using a nested primer pair. For this second PCR, the following degenerate primers were used:

ACGCTGAATTCGCGAYAAAYATHGARTAYATHAC (SEQ ID

5 NO:20)

ACGCTAAGCTTGCNGARTAYTCRAANGTRAA (SEQ ID NO:21)

10 In these primer sequences, R represents A or G; Y represents T or C; H represents A or C or T; N represents A or T or C or G.

This second PCR was performed using the same
15 reaction mix and cycling parameters as for the first PCR. Analysis of this second PCR by agarose gel electrophoresis revealed the amplification of an approximately 1 kb DNA fragment. This DNA fragment was gel purified, and then digested with restriction
20 endonucleases *EcoRI* and *HindIII*, and ligated into the cloning/sequencing vector mp19 (Boehringer Mannheim Corporation) for sequencing.

An oligonucleotide identical to a small portion of the sequence of the DNA fragment was
25 generated and used to isolate the full-length AFM cDNA from a human liver cDNA library (Clontech, cat no. HL1115a). The sequence of this oligonucleotide was:

TATGTGCTATGGAGGGGC (SEQ ID NO:22)

30

Positive clones from this library screening were purified using standard procedures and then re-screened with the same oligonucleotide probe, and a single clone (called 17AFM) containing an approximately
35 2.3 kb insert was selected. This clone was inserted into the vector pGem3Z for sequencing to confirm that it

- 43 -

encoded the full-length cDNA for human AFM. The nucleic acid sequence and translated amino acid sequence for this clone are set forth in Figure 4.

To prepare a transgenic mouse containing the transgene human AFM, the AFM cDNA clone was removed from the vector by digesting with *EcoRI*. The cDNA was then inserted into the vector plivBSK (described in Example I). This vector containing the AFM cDNA, was transformed into *E. coli* strain DH5 alpha for amplification. After culturing the cells overnight, the plasmid was isolated and purified using the standard alkaline lysis method, followed by cesium chloride centrifugation.

After centrifugation, the plasmid was digested with the restriction enzymes *SpeI*, *XhoI*, and *ScaI*, and the DNA fragment containing the HCR, ApoE promoter and first intron, AFM cDNA and the SV40 polyA sequence was isolated. This DNA was gel purified and prepared for microinjection as described in Example I.

Microinjection and implantation of embryos into pseudopregnant mice were as described in Example I.

All literature cited herein is expressly incorporated by reference.

25

- 44 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Amgen Inc.
- (ii) TITLE OF INVENTION: Tissue Specific Transgene Expression
- (iii) NUMBER OF SEQUENCES: 24
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Amgen Inc., U.S. Patent Operations/NAO
 - (B) STREET: 1840 Dehavilland Drive
 - (C) CITY: Thousand Oaks
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 91320-1789
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 774 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCAGGCTC AGAGGCACAC AGGAGTTTCT GGGCTCACCC TGCCCCCTTC CAACCCCTCA	60
GTTCCCATCC TCCAGCAGCT GTTTGTGTGC TGCCTCTGAA GTCCACACTG AACAAACTTC	120
AGCCTACTCA TGTCCCTAAA ATGGGCAAAC ATTGCAAGCA GCAAACAGCA AACACACAGC	180
CCTCCCTGCC TGCTGACCTT GGAGCTGGGG CAGAGGTCAG AGACCTCTCT GGGCCCATGC	240
CACCTCCAAC ATCCACTCGA CCCCTTGGAA TTTCGGTGGA GAGGAGCAGA GGTTCCTCTG	300
GCGTGGTTTA GGTAGTGTGA GAGGGTCCGG GTTCAAACC ACTTGCTGGG TGGGGAGTCG	360

- 45 -

TCAGTAAGTG GCTATGCCCC GACCCCGAAG CCTGTTTCCC CATCTGTACA ATGGAATGA 420
TAAAGACGCC CATCTGATAG GGTTTTTG TG GCAAATAAAC ATTTGGTTTT TTTGTTTTGT 480
TTGTTTTGT TTTTGAGAT GGAGGTTTGC TCTGTCGCCC AGGCTGGAGT GCAGTGACAC 540
AATCTCATCT CACCACAACC TTCCCCTGCC TCAGCCTCCC AAGTAGCTGG GATTACAAGC 600
ATGTGCCACC ACACCTGGCT AATTTTCTAT TTTTAGTAGA GACGGGTTTC TCCATGTTGG 660
TCAGCCTCAG CCTCCCAAGT AACTGGGATT ACAGGCCTGT GCCACCACAC CCGGCTAATT 720
TTTTCTATTT TTGACAGGGA CGGGGTTTCA CCATGTTGGT CAGGCTGGTC TAGA 774

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTAGAAAG CTTAATTCAG TC 22

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCCCGCGGG GAAGAGCGCA GAGCTCGG 28

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 46 -

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
CTCTAGAAAG CTTAATTCAG TC 22
- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CTGGATCTCG AGGTACCCGG GGATCATAAT C 31
- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
ATGTCGACMW CSVTGCMCCH RYMYSMYCYA 30
- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
GGACTAGTCC AGAGCACACA AGCTTCTAG 29
- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid

- 47 -

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATAAGAATGC GGCCGCTAAA CTATTGCATC TGGCAACCC

39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCTCTAGAA AGAGCTGGGA C

21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCCGTGTTC CATTATGAG C

21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAATCTACAA TTCACAGA

18

- 48 -

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTAAGTTATT GCCATAGG

18

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AACAAAGCTT CTACAATTCA CAGATAGGA

29

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACAAAGATCT TAAGTTATTG CCATAGG

27

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 49 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGATCGTAAG CTTGGTCAAT GACCTAGGAG TAAC

34

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGATCGTAAG CTTGCGGATC CTAAGTTATT GCC

33

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTGTSYCTSC TSNTSMTWGT WGCYGSCT

28

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACGCTGAATT CGCCARAART TYATHGARGA YAA

33

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:

- 50 -

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ACGCTAAGCT TGCRTCYTTR TADATYTGNA CDAT

34

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ACGCTGAATT CGCGAYAAYA THGARTAYAT HAC

33

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACGCTAAGCT TGCNGARTAY TCRAANGTRA A

31

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

- 51 -

TATGTGCTAT GGAGGGGC

18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2287 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 318..2117

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 381..2114

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
- (B) LOCATION: 318..380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCCGAGTCT CTGCGCCTTC ACATAGTTGT CACAGGACTA AAGCAAATTG ATCCAGGGGG	60
AAACACTGTA GACCGTGTAT ATAAAAACAC TCTATAAACT GCAATGCTCA ATTCTTAGTA	120
TAACTATTGT TGTGTATTG ATATTTATTA GTATTGGTGC TCACAAAAG AGTCTAAATT	180
CCATAAGTCT TTATATTCAG GCTACTCTTT ATTTTGAAG ACTCATTTC TATCACCTTT	240
TTCTATTTTA CTCCATATTG AGGCCTCATA AATCCAATTT TTTATTTCTT TCTTTGTAA	300
ATGTGGTTTC TACAAAG ATG AAA CTA CTA AAA CTT ACA GGT TTT ATT TTT	350
Met Lys Leu Leu Lys Leu Thr Gly Phe Ile Phe	
-21 -20 -15	
TTC TTG TTT TTT TTG ACT GAA TCC CTA ACC CTG CCC ACA CAA CCT CGG	398
Phe Leu Phe Phe Leu Thr Glu Ser Leu Thr Leu Pro Thr Gln Pro Arg	
-10 -5 1 5	
GAT ATA GAG AAC TTC AAT AGT ACT CAA AAA TTT ATA GAA GAT AAT ATT	446
Asp Ile Glu Asn Phe Asn Ser Thr Gln Lys Phe Ile Glu Asp Asn Ile	
10 15 20	
GAA TAC ATC ACC ATC ATT GCA TTT GCT CAG TAT GTT CAG GAA GCA ACC	494
Glu Tyr Ile Thr Ile Ile Ala Phe Ala Gln Tyr Val Gln Glu Ala Thr	
25 30 35	
TTT GAA GAA ATG GAA AAG CTG GTG AAA GAC ATG GTA GAA TAC AAA GAC	542
Phe Glu Glu Met Glu Lys Leu Val Lys Asp Met Val Glu Tyr Lys Asp	
40 45 50	

- 52 -

AGA TGT ATG GCT GAC AAG ACG CTC CCA GAG TGT TCA AAA TTA CCT AAT	590
Arg Cys Met Ala Asp Lys Thr Leu Pro Glu Cys Ser Lys Leu Pro Asn	
55 60 65 70	
AAT GTT TTA CAG GAA AAA ATA TGT GCT ATG GAG GGG CTG CCA CAA AAG	638
Asn Val Leu Gln Glu Lys Ile Cys Ala Met Glu Gly Leu Pro Gln Lys	
75 80 85	
CAT AAT TTC TCA CAC TGC TGC AGT AAG GTT GAT GCT CAA AGA AGA CTC	686
His Asn Phe Ser His Cys Cys Ser Lys Val Asp Ala Gln Arg Arg Leu	
90 95 100	
TGT TTC TTC TAT AAC AAG AAA TCT GAT GTG GGA TTT CTG CCT CCT TTC	734
Cys Phe Tyr Asn Lys Lys Ser Asp Val Gly Phe Leu Pro Pro Phe	
105 110 115	
CCT ACC CTG GAT CCC GAA GAG AAA TGC CAG GCT TAT GAA AGT AAC AGA	782
Pro Thr Leu Asp Pro Glu Glu Lys Cys Gln Ala Tyr Glu Ser Asn Arg	
120 125 130	
GAA TCC CTT TTA AAT CAC TTT TTA TAT GAA GTT GCC AGA AGG AAC CCA	830
Glu Ser Leu Leu Asn His Phe Leu Tyr Glu Val Ala Arg Arg Asn Pro	
135 140 145 150	
TTT GTC TTC GCC CCT ACA CTT CTA ACT GTT GCT GTT CAT TTT GAG GAG	878
Phe Val Phe Ala Pro Thr Leu Leu Thr Val Ala Val His Phe Glu Glu	
155 160 165	
GTG GCC AAA TCA TGT TGT GAA GAA CAA AAC AAA GTC AAC TGC CTT CAA	926
Val Ala Lys Ser Cys Cys Glu Glu Gln Asn Lys Val Asn Cys Leu Gln	
170 175 180	
ACA AGG GCA ATA CCT GTC ACA CAA TAT TTA AAA GCA TTT TCT TCT TAT	974
Thr Arg Ala Ile Pro Val Thr Gln Tyr Leu Lys Ala Phe Ser Ser Tyr	
185 190 195	
CAA AAA CAT GTC TGT GGG GCA CTT TTG AAA TTT GGA ACC AAA GTT GTA	1022
Gln Lys His Val Cys Gly Ala Leu Leu Lys Phe Gly Thr Lys Val Val	
200 205 210	
CAC TTT ATA TAT ATT GCG ATA CTC AGT CAA AAA TTC CCC AAG ATT GAA	1070
His Phe Ile Tyr Ile Ala Ile Leu Ser Gln Lys Phe Pro Lys Ile Glu	
215 220 225 230	
TTT AAG GAG CTT ATT TCT CTT GTA GAA GAT GTT TCT TCC AAC TAT GAT	1118
Phe Lys Glu Leu Ile Ser Leu Val Glu Asp Val Ser Ser Asn Tyr Asp	
235 240 245	
GGA TGC TGT GAA GGG GAT GTT GTG CAG TGC ATC CGT GAC ACG AGC AAG	1166
Gly Cys Cys Glu Gly Asp Val Val Gln Cys Ile Arg Asp Thr Ser Lys	
250 255 260	
GTT ATG AAC CAT ATT TGT TCA AAA CAA GAT TCT ATC TCC AGC AAA ATC	1214
Val Met Asn His Ile Cys Ser Lys Gln Asp Ser Ile Ser Ser Lys Ile	
265 270 275	
AAA GAG TGC TGT GAA AAG AAA ATA CCA GAG CGC GGC CAG TGC ATA ATT	1262

- 53 -

Lys	Glu	Cys	Cys	Glu	Lys	Lys	Ile	Pro	Glu	Arg	Gly	Gln	Cys	Ile	Ile	
280						285					290					
AAC	TCA	AAC	AAA	GAT	GAT	AGA	CCA	AAG	GAT	TTA	TCT	CTA	AGA	GAA	GGA	1310
Asn	Ser	Asn	Lys	Asp	Asp	Arg	Pro	Lys	Asp	Leu	Ser	Leu	Arg	Glu	Gly	
295				300					305						310	
AAA	TTT	ACT	GAC	AGT	GAA	AAT	GTG	TGT	CAA	GAA	CGA	GAT	GCT	GAC	CCA	1358
Lys	Phe	Thr	Asp	Ser	Glu	Asn	Val	Cys	Gln	Glu	Arg	Asp	Ala	Asp	Pro	
				315					320					325		
GAC	ACC	TTC	TTT	GCG	AAG	TTT	ACT	TTT	GAA	TAC	TCA	AGG	AGA	CAT	CCA	1406
Asp	Thr	Phe	Phe	Ala	Lys	Phe	Thr	Phe	Glu	Tyr	Ser	Arg	Arg	His	Pro	
			330					335						340		
GAC	CTG	TCT	ATA	CCA	GAG	CTT	TTA	AGA	ATT	GTT	CAA	ATA	TAC	AAA	GAT	1454
Asp	Leu	Ser	Ile	Pro	Glu	Leu	Leu	Arg	Ile	Val	Gln	Ile	Tyr	Lys	Asp	
			345				350					355				
CTC	CTG	AGA	AAT	TGC	TGC	AAC	ACA	GAA	AAC	CCT	CCA	GGT	TGT	TAC	CGT	1502
Leu	Leu	Arg	Asn	Cys	Cys	Asn	Thr	Glu	Asn	Pro	Pro	Gly	Cys	Tyr	Arg	
			360			365					370					
TAC	GCG	GAA	GAC	AAA	TTC	AAT	GAG	ACA	ACT	GAG	AAA	AGC	CTC	AAG	ATG	1550
Tyr	Ala	Glu	Asp	Lys	Phe	Asn	Glu	Thr	Thr	Glu	Lys	Ser	Leu	Lys	Met	
375					380					385					390	
GTA	CAA	CAA	GAA	TGT	AAA	CAT	TTC	CAG	AAT	TTG	GGG	AAG	GAT	GGT	TTG	1598
Val	Gln	Gln	Glu	Cys	Lys	His	Phe	Gln	Asn	Leu	Gly	Lys	Asp	Gly	Leu	
				395				400						405		
AAA	TAC	CAT	TAC	CTC	ATC	AGG	CTC	ACG	AAG	ATA	GCT	CCC	CAA	CTC	TCC	1646
Lys	Tyr	His	Tyr	Leu	Ile	Arg	Leu	Thr	Lys	Ile	Ala	Pro	Gln	Leu	Ser	
			410					415					420			
ACT	GAA	GAA	CTG	GTG	TCT	CTT	GGC	GAG	AAA	ATG	GTG	ACA	GCT	TTC	ACT	1694
Thr	Glu	Glu	Leu	Val	Ser	Leu	Gly	Glu	Lys	Met	Val	Thr	Ala	Phe	Thr	
			425				430					435				
ACT	TGC	TGT	ACG	CTA	AGT	GAA	GAG	TTT	GCC	TGT	GTT	GAT	AAT	TTG	GCA	1742
Thr	Cys	Cys	Thr	Leu	Ser	Glu	Glu	Phe	Ala	Cys	Val	Asp	Asn	Leu	Ala	
			440			445				450						
GAT	TTA	GTT	TTT	GGA	GAG	TTA	TGT	GGA	GTA	AAT	GAA	AAT	CGA	ACT	ATC	1790
Asp	Leu	Val	Phe	Gly	Glu	Leu	Cys	Gly	Val	Asn	Glu	Asn	Arg	Thr	Ile	
455					460				465						470	
AAC	CCT	GCT	GTG	GAC	CAC	TGC	TGT	AAA	ACA	AAC	TTT	GCC	TTC	AGA	AGG	1838
Asn	Pro	Ala	Val	Asp	His	Cys	Cys	Lys	Thr	Asn	Phe	Ala	Phe	Arg	Arg	
				475				480						485		
CCC	TGC	TTT	GAG	AGT	TTG	AAA	GCT	GAT	AAA	ACA	TAT	GTG	CCT	CCA	CCT	1886
Pro	Cys	Phe	Glu	Ser	Leu	Lys	Ala	Asp	Lys	Thr	Tyr	Val	Pro	Pro	Pro	
			490				495						500			
TTC	TCT	CAA	GAT	TTA	TTT	ACC	TTT	CAC	GCA	GAC	ATG	TGT	CAA	TCT	CAG	1934
Phe	Ser	Gln	Asp	Leu	Phe	Thr	Phe	His	Ala	Asp	Met	Cys	Gln	Ser	Gln	
			505				510					515				

- 54 -

AAT GAG GAG CTT CAG AGG AAG ACA GAC AGG TTT CTT GTC AAC TTA GTG	1982
Asn Glu Glu Leu Gln Arg Lys Thr Asp Arg Phe Leu Val Asn Leu Val	
520 525 530	
AAG CTG AAG CAT GAA CTC ACA GAT GAA GAG CTG CAG TCT TTG TTT ACA	2030
Lys Leu Lys His Glu Leu Thr Asp Glu Glu Leu Gln Ser Leu Phe Thr	
535 540 545 550	
AAT TTC GCA AAT GTA GTG GAT AAG TGC TGC AAA GCA GAG AGT CCT GAA	2078
Asn Phe Ala Asn Val Val Asp Lys Cys Cys Lys Ala Glu Ser Pro Glu	
555 560 565	
GTC TGC TTT AAT GAA GAG AGT CCA AAA ATT GGC AAC TGAAGCCAGC	2124
Val Cys Phe Asn Glu Glu Ser Pro Lys Ile Gly Asn	
570 575	
TGCTGGAGAT ATGTAAAGAA AAAAGCACCA AAGGGAAGGC TTCCTATCTG TGTGGTGATG	2184
AATCGCATTT CTGAGAACA AAATAAAAGG ATTTTCTGT AACTGTCACC TGAAATAATA	2244
CATTGCAGCA AGCAATAAAC ACAACATTTT GTAAAGTTAA AAA	2287

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 599 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Lys Leu Leu Lys Leu Thr Gly Phe Ile Phe Phe Leu Phe Phe Leu	
-21 -20 -15 -10	
Thr Glu Ser Leu Thr Leu Pro Thr Gln Pro Arg Asp Ile Glu Asn Phe	
-5 1 5 10	
Asn Ser Thr Gln Lys Phe Ile Glu Asp Asn Ile Glu Tyr Ile Thr Ile	
15 20 25	
Ile Ala Phe Ala Gln Tyr Val Gln Glu Ala Thr Phe Glu Glu Met Glu	
30 35 40	
Lys Leu Val Lys Asp Met Val Glu Tyr Lys Asp Arg Cys Met Ala Asp	
45 50 55	
Lys Thr Leu Pro Glu Cys Ser Lys Leu Pro Asn Asn Val Leu Gln Glu	
60 65 70 75	
Lys Ile Cys Ala Met Glu Gly Leu Pro Gln Lys His Asn Phe Ser His	
80 85 90	
Cys Cys Ser Lys Val Asp Ala Gln Arg Arg Leu Cys Phe Phe Tyr Asn	
95 100 105	

- 55 -

Lys Lys Ser Asp Val Gly Phe Leu Pro Pro Phe Pro Thr Leu Asp Pro
 110 115 120
 Glu Glu Lys Cys Gln Ala Tyr Glu Ser Asn Arg Glu Ser Leu Leu Asn
 125 130 135
 His Phe Leu Tyr Glu Val Ala Arg Arg Asn Pro Phe Val Phe Ala Pro
 140 145 150 155
 Thr Leu Leu Thr Val Ala Val His Phe Glu Glu Val Ala Lys Ser Cys
 160 165 170
 Cys Glu Glu Gln Asn Lys Val Asn Cys Leu Gln Thr Arg Ala Ile Pro
 175 180 185
 Val Thr Gln Tyr Leu Lys Ala Phe Ser Ser Tyr Gln Lys His Val Cys
 190 195 200
 Gly Ala Leu Leu Lys Phe Gly Thr Lys Val Val His Phe Ile Tyr Ile
 205 210 215
 Ala Ile Leu Ser Gln Lys Phe Pro Lys Ile Glu Phe Lys Glu Leu Ile
 220 225 230 235
 Ser Leu Val Glu Asp Val Ser Ser Asn Tyr Asp Gly Cys Cys Glu Gly
 240 245 250
 Asp Val Val Gln Cys Ile Arg Asp Thr Ser Lys Val Met Asn His Ile
 255 260 265
 Cys Ser Lys Gln Asp Ser Ile Ser Ser Lys Ile Lys Glu Cys Cys Glu
 270 275 280
 Lys Lys Ile Pro Glu Arg Gly Gln Cys Ile Ile Asn Ser Asn Lys Asp
 285 290 295
 Asp Arg Pro Lys Asp Leu Ser Leu Arg Glu Gly Lys Phe Thr Asp Ser
 300 305 310 315
 Glu Asn Val Cys Gln Glu Arg Asp Ala Asp Pro Asp Thr Phe Phe Ala
 320 325 330
 Lys Phe Thr Phe Glu Tyr Ser Arg Arg His Pro Asp Leu Ser Ile Pro
 335 340 345
 Glu Leu Leu Arg Ile Val Gln Ile Tyr Lys Asp Leu Leu Arg Asn Cys
 350 355 360
 Cys Asn Thr Glu Asn Pro Pro Gly Cys Tyr Arg Tyr Ala Glu Asp Lys
 365 370 375
 Phe Asn Glu Thr Thr Glu Lys Ser Leu Lys Met Val Gln Gln Glu Cys
 380 385 390 395
 Lys His Phe Gln Asn Leu Gly Lys Asp Gly Leu Lys Tyr His Tyr Leu
 400 405 410

- 56 -

Ile Arg Leu Thr Lys Ile Ala Pro Gln Leu Ser Thr Glu Glu Leu Val
 415 420 425
 Ser Leu Gly Glu Lys Met Val Thr Ala Phe Thr Thr Cys Cys Thr Leu
 430 435 440
 Ser Glu Glu Phe Ala Cys Val Asp Asn Leu Ala Asp Leu Val Phe Gly
 445 450 455
 Glu Leu Cys Gly Val Asn Glu Asn Arg Thr Ile Asn Pro Ala Val Asp
 460 465 470 475
 His Cys Cys Lys Thr Asn Phe Ala Phe Arg Arg Pro Cys Phe Glu Ser
 480 485 490
 Leu Lys Ala Asp Lys Thr Tyr Val Pro Pro Pro Phe Ser Gln Asp Leu
 495 500 505
 Phe Thr Phe His Ala Asp Met Cys Gln Ser Gln Asn Glu Glu Leu Gln
 510 515 520
 Arg Lys Thr Asp Arg Phe Leu Val Asn Leu Val Lys Leu Lys His Glu
 525 530 535
 Leu Thr Asp Glu Glu Leu Gln Ser Leu Phe Thr Asn Phe Ala Asn Val
 540 545 550 555
 Val Asp Lys Cys Cys Lys Ala Glu Ser Pro Glu Val Cys Phe Asn Glu
 560 565 570
 Glu Ser Pro Lys Ile Gly Asn
 575

- 57 -

We Claim:

1. A nucleic acid sequence comprising an HCR enhancer operably linked to a promoter and a transgene.

5

2. The nucleic acid sequence of claim 1 wherein the promoter is selected from the group of promoters consisting of: ApoA-I, ApoA-II, ApoA-III, ApoA-IV, ApoB-48, ApoB-100, ApoC-I, ApoC-II, ApoC-III, ApoE, albumin, alpha feto protein, PEPCK, transthyretin, SV40, CMV, and TK.

10

3. The nucleic acid sequence of claim 1 further comprising an intron and a polyadenylation sequence, wherein the HCR enhancer, the promoter, the intron, the transgene and the polyadenylation sequence are all operably linked such that the coding sequence of the transgene may be expressed.

15

4. The nucleic acid sequence of claim 1 wherein the transgene comprises a nucleic acid encoding a polypeptide involved in the immune response, hematopoiesis, inflammation, cell growth and proliferation, cell lineage differentiation, or the stress response.

20

25

5. The nucleic acid sequence of claim 4 wherein the transgene is selected from the group consisting of: interleukin 1, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, ENA-78, interferon- α , interferon- β , interferon- γ , granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating

30

35

- 58 -

factor, stem cell factor, keratinocyte growth factor, AFM, MCP-1 and TNF, and fragments thereof.

6. The nucleic acid sequence of claim 5
5 comprising the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and the coding sequence of the transgene human IL-8.

10 7. The nucleic acid sequence of claim 5 comprising the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and the coding sequence of the transgene human KGF.

15 8. The nucleic acid sequence of claim 5 comprising the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2,
20 and the coding sequence of the transgene human MCP-1.

9. The nucleic acid sequence of claim 5 comprising the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1
25 and at its 3' end to a portion of the human ApoE exon 2, and the coding sequence of the transgene human AFM.

10. A non-human mammal or its progeny containing a nucleic acid sequence comprising an HCR
30 enhancer operably linked to a promoter and a transgene.

11. The non-human mammal of claim 10 wherein the promoter is selected from the group of promoters consisting of: ApoA-I, ApoA-II, ApoA-III, ApoA-IV, ApoB-
35 48, ApoB-100, ApoC-I, ApoC-II, ApoC-III, ApoE, albumin,

- 59 -

alpha feto protein, PEPCK, transthyretin, SV40, CMV, and TK.

12. The non-human mammal of claim 11 wherein the nucleic acid sequence further comprises an
5 intron, at least a portion of the coding sequence of a transgene, and a polyadenylation sequence, and wherein the HCR enhancer, the promoter, the intron, the transgene, and the polyadenylation sequence are all operably linked such that the transgene may be
10 expressed.

13. The non-human mammal of claim 11 wherein the transgene comprises a nucleic acid encoding a polypeptide involved in the immune response,
15 hematopoiesis, inflammation, cell growth and proliferation, cell lineage differentiation, or the stress response.

14. The non-human mammal of claim 13 wherein
20 the transgene is selected from the group consisting of: interleukin 1, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, ENA-78, interferon- α ,
25 interferon- β , interferon- γ , granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, stem cell factor, keratinocyte growth factor, AFM, MCP-1 and TNF, and fragments thereof.

30
15. The non-human mammal of claim 14 wherein the nucleic acid sequence comprises the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion
35 of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human IL-8.

- 60 -

16. The non-human mammal of claim 14 wherein the nucleic acid sequence comprises the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human KGF.

17. The non-human mammal of claim 14 wherein the nucleic acid sequence comprises the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human MCP-1.

18. The non-human mammal of claim 14 wherein the nucleic acid sequence comprises the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human AFM.

19. The non-human mammal of claims 10, 11, 12, 13, 14, 15, 16, 17 or 18 that is a rodent.

20. The rodent of claim 19 that is a mouse.

21. A vector comprising the nucleic acid sequence of claim 1.

22. A vector comprising the nucleic acid sequence of claim 3.

23. A vector comprising the nucleic acid sequence of claim 5.

- 61 -

24. A vector comprising the nucleic acid
sequence of claim 6.

25. A vector comprising the nucleic acid
5 sequence of claim 7.

26. A prokaryotic cell containing the vector
of claims 21, 22, 23, 24, or 25.

10 27. A eukaryotic cell containing the vector
of claims 21, 22, 23, 24 or 25.

28. A prokaryotic cell containing the nucleic
acid sequence of claims 1, 2, 3, 4, 5, 6, 7, or 8.
15

29. A eukaryotic cell containing the nucleic
acid sequence of claims 1, 2, 3, 4, 5, 6, 7, or 8.

1 / 8

FIG. 1

CTGCAGGCTC AGAGGCACAC AGGAGTTTCT GGGCTCACCC TGCCCCCTTC
CAACCCCTCA GTTCCCATCC TCCAGCAGCT GTTGTGTGC TGCCTCTGAA
GTCCACACTG AACAACTTC AGCCTACTCA TGTCCCTAAA ATGGGCAAAC
ATTGCAAGCA GCAAACAGCA AACACACAGC CCTCCCTGCC TGCTGACCTT
GGAGCTGGGG CAGAGGTCAG AGACCTCTCT GGGCCCATGC CACCTCCAAC
ATCCACTCGA CCCCTTGGAA TTTCGGTGGA GAGGAGCAGA GGTGTCTCTG
GCGTGGTTTA GGTAGTGTGA GAGGGTCCGG GTTCAAAACC ACTTGCTGGG
TGGGGAGTCG TCAGTAAGTG GCTATGCCCC GACCCCGAAG CCTGTTTCCC
CATCTGTACA ATGGAAATGA TAAAGACGCC CATCTGATAG GGTTTTTGTG
GCAAATAAAC ATTTGGTTTT TTTGTTTTGT TTTGTTTTGT TTTTGTAGAT
GGAGGTTTGC TCTGTCGCCC AGGCTGGAGT GCAGTGACAC AATCTCATCT
CACCACAACC TTCCCCTGCC TCAGCCTCCC AAGTAGCTGG GATTACAAGC
ATGTGCCACC ACACCTGGCT AATTTTCTAT TTTTAGTAGA GACGGGTTTC
TCCATGTTGG TCAGCCTCAG CCTCCCAAGT AACTGGGATT ACAGGCCTGT
GCCACCACAC CCGGCTAATT TTTTCTATTT TTGACAGGGA CGGGGTTTCA
CCATGTTGGT CAGGCTGGTC TAGA

FIG. 2A

2 / 8

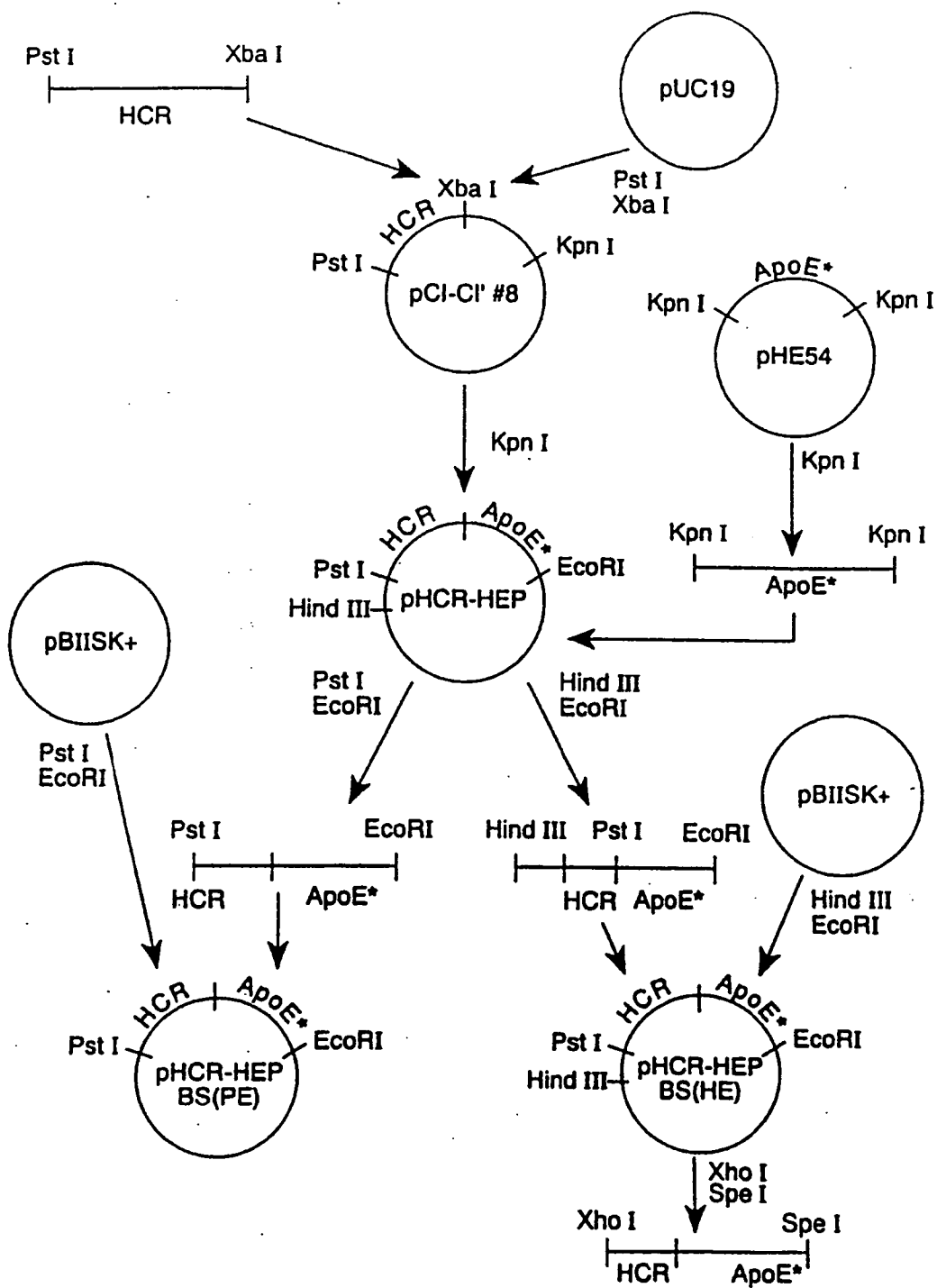
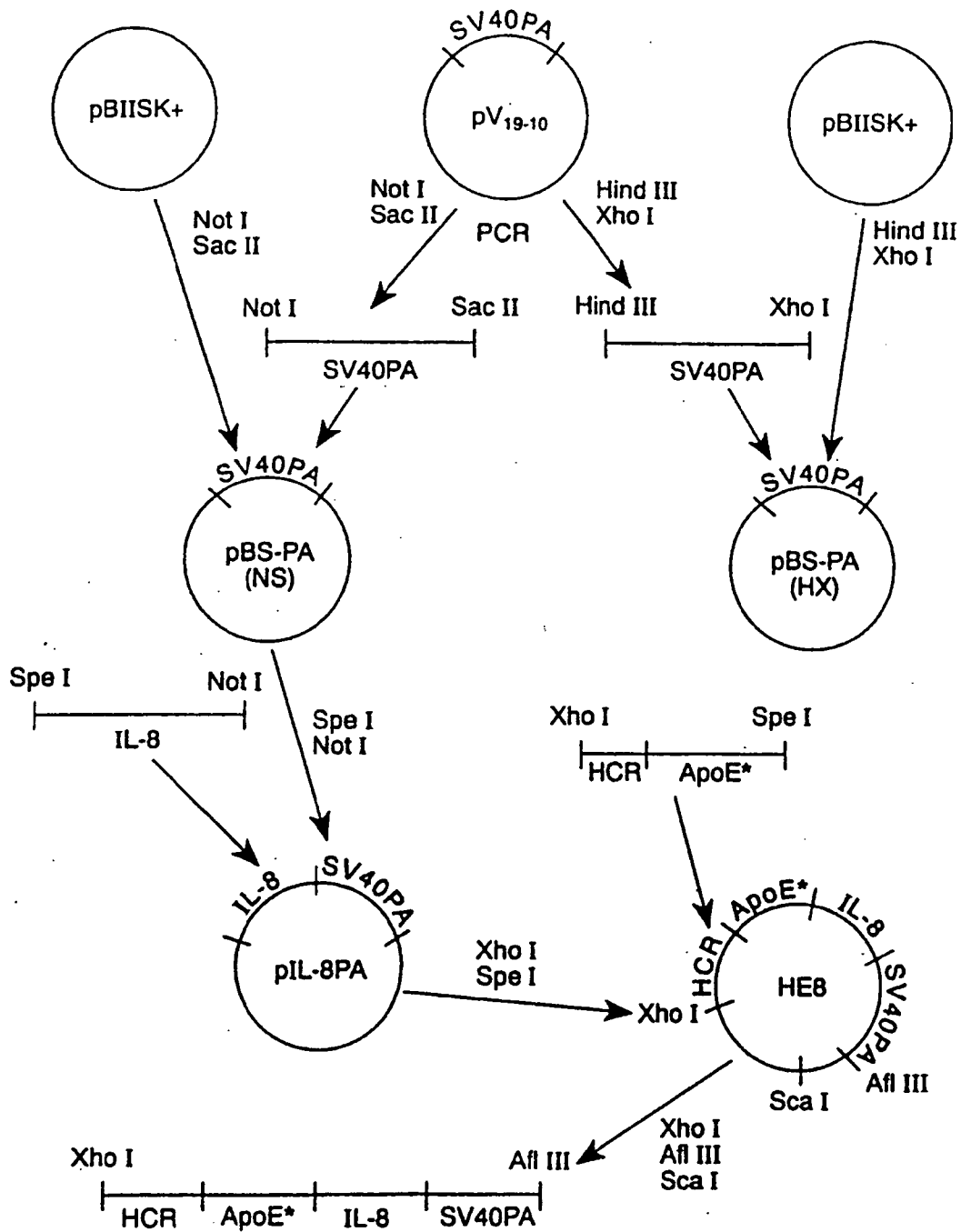
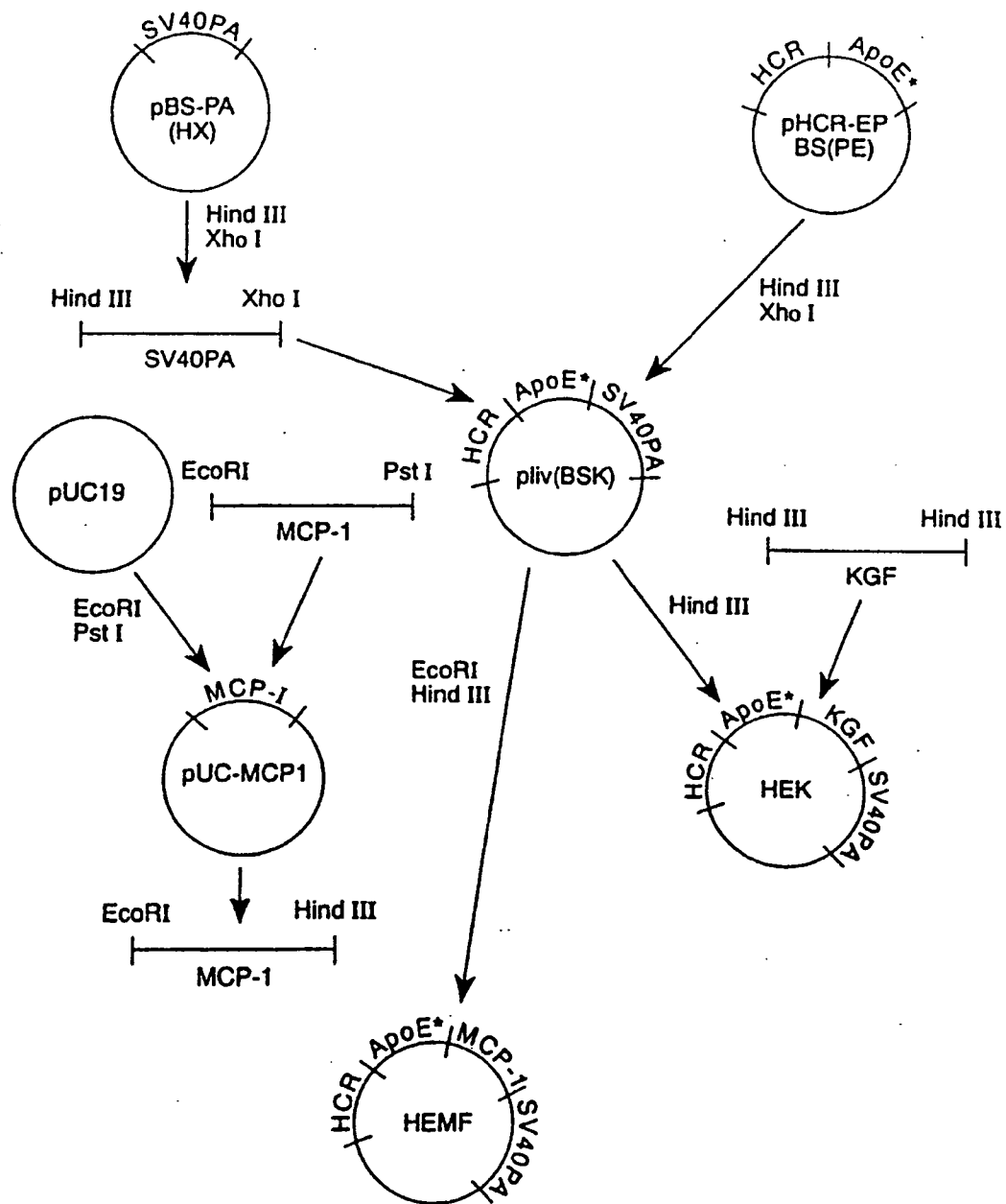


FIG. 2B 3 / 8



4 / 8

FIG. 2C



5 / 8

FIG.3A

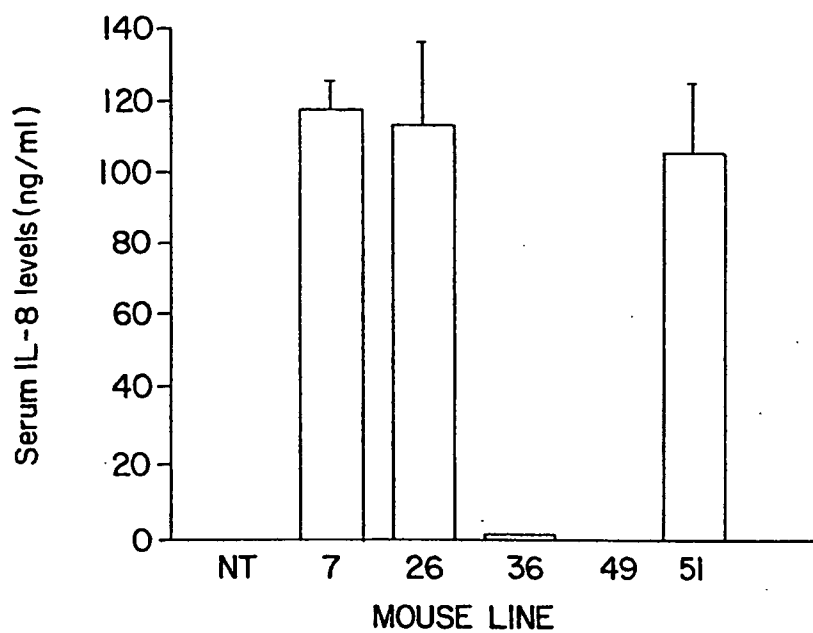
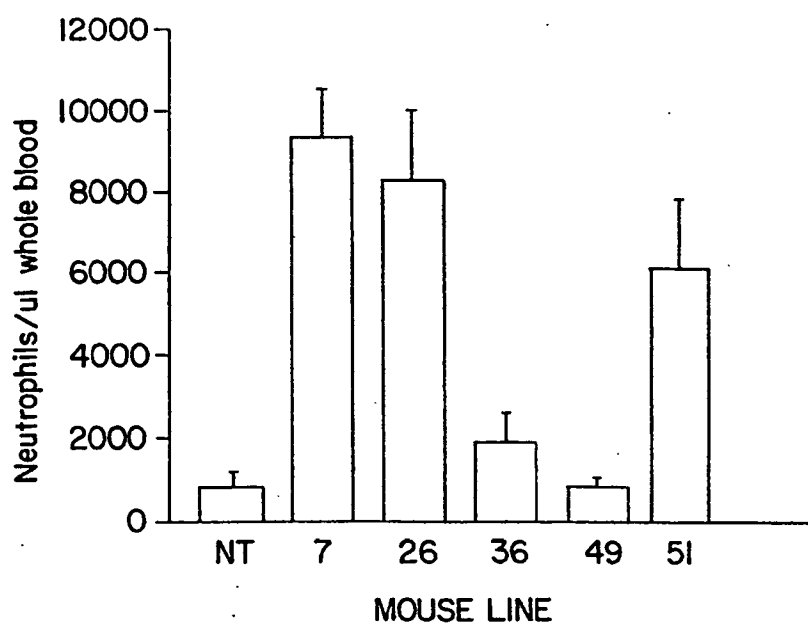


FIG.3B



6/8

FIG. 4A

CCCCAGAGTCT CTGGGCCTTC ACATAGTTGT CACAGGACTA AAGCAAATTG ATCCAGGGGG
 AAACACTGTA GACCGTGTAT ATAAAAACAC TCTATAAACT GCAATGCTCA ATTCTTAGTA
 TAACTATTGT TGTGTATTG ATATTTATTA GTATTGGTGC TCACAAAAAG AGTCTAAATT
 CCATAAGTCT TTATATTCAG GCTACTCTTT ATTTTGGAAA ACTCATTTTC TATCACCTTT
 TTCTATTTTA CTCCATATTG AGGCCTCATA AATCCAATTT TTTATTCTT TCTTTTGTA
 ATGTGGTTTC TACAAAG ATG AAA CTA CTA AAA CTT ACA GGT TTT ATT TTT
 Met Lys Leu Leu Lys Leu Thr Gly Phe Ile Phe
 -21 -20 -15
 TTC TTG TTT TTT TTG ACT GAA TCC CTA ACC CTG CCC ACA CAA CCT CGG
 Phe Leu Phe Phe Leu Thr Glu Ser Leu Thr Leu Pro Thr Gln Pro Arg
 -10 -5 1 5
 GAT ATA GAG AAC TTC AAT AGT ACT CAA AAA TTT ATA GAA GAT AAT ATT
 Asp Ile Glu Asn Phe Asn Ser Thr Gln Lys Phe Ile Glu Asp Asn Ile
 10 15 20
 GAA TAC ATC ACC ATC ATT GCA TTT GCT CAG TAT GTT CAG GAA GCA ACC
 Glu Tyr Ile Thr Ile Ile Ala Phe Ala Gln Tyr Val Gln Glu Ala Thr
 25 30 35
 TTT GAA GAA ATG GAA AAG CTG GTG AAA GAC ATG GTA GAA TAC AAA GAC
 Phe Glu Glu Met Glu Lys Leu Val Lys Asp Met Val Glu Tyr Lys Asp
 40 45 50
 AGA TGT ATG GCT GAC AAG ACG CTC CCA GAG TGT TCA AAA TTA CCT AAT
 Arg Cys Met Ala Asp Lys Thr Leu Pro Glu Cys Ser Lys Leu Pro Asn
 55 60 65 70
 AAT GTT TTA CAG GAA AAA ATA TGT GCT ATG GAG GGG CTG CCA CAA AAG
 Asn Val Leu Gln Glu Lys Ile Cys Ala Met Glu Gly Leu Pro Gln Lys
 75 80 85
 CAT AAT TTC TCA CAC TGC TGC AGT AAG GTT GAT GCT CAA AGA AGA CTC
 His Asn Phe Ser His Cys Cys Ser Lys Val Asp Ala Gln Arg Arg Leu
 90 95 100
 TGT TTC TTC TAT AAC AAG AAA TCT GAT GTG GGA TTT CTG CCT CCT TTC
 Cys Phe Phe Tyr Asn Lys Lys Ser Asp Val Gly Phe Leu Pro Pro Phe
 105 110 115
 CCT ACC CTG GAT CCC GAA GAG AAA TGC CAG GCT TAT GAA AGT AAC AGA
 Pro Thr Leu Asp Pro Glu Glu Lys Cys Gln Ala Tyr Glu Ser Asn Arg
 120 125 130
 GAA TCC CTT TTA AAT CAC TTT TTA TAT GAA GTT GCC AGA AGG AAC CCA
 Glu Ser Leu Leu Asn His Phe Leu Tyr Glu Val Ala Arg Arg Asn Pro
 135 140 145 150
 TTT GTC TTC GCC CCT ACA CTT CTA ACT GTT GCT GTT CAT TTT GAG GAG

7 / 8

FIG. 4B

Phe	Val	Phe	Ala	Pro	Thr	Leu	Leu	Thr	Val	Ala	Val	His	Phe	Glu	Glu
				155					160					165	
GTG	GCC	AAA	TCA	TGT	TGT	GAA	GAA	CAA	AAC	AAA	GTC	AAC	TGC	CTT	CAA
Val	Ala	Lys	Ser	Cys	Cys	Glu	Glu	Gln	Asn	Lys	Val	Asn	Cys	Leu	Gln
			170					175					180		
ACA	AGG	GCA	ATA	CCT	GTC	ACA	CAA	TAT	TTA	AAA	GCA	TTT	TCT	TCT	TAT
Thr	Arg	Ala	Ile	Pro	Val	Thr	Gln	Tyr	Leu	Lys	Ala	Phe	Ser	Ser	Tyr
		185					190					195			
CAA	AAA	CAT	GTC	TGT	GGG	GCA	CTT	TTG	AAA	TTT	GGA	ACC	AAA	GTT	GTA
Gln	Lys	His	Val	Cys	Gly	Ala	Leu	Leu	Lys	Phe	Gly	Thr	Lys	Val	Val
	200					205					210				
CAC	TTT	ATA	TAT	ATT	GCG	ATA	CTC	AGT	CAA	AAA	TTC	CCC	AAG	ATT	GAA
His	Phe	Ile	Tyr	Ile	Ala	Ile	Leu	Ser	Gln	Lys	Phe	Pro	Lys	Ile	Glu
215					220					225					230
TTT	AAG	GAG	CTT	ATT	TCT	CTT	GTA	GAA	GAT	GTT	TCT	TCC	AAC	TAT	GAT
Phe	Lys	Glu	Leu	Ile	Ser	Leu	Val	Glu	Asp	Val	Ser	Ser	Asn	Tyr	Asp
			235					240						245	
GGA	TGC	TGT	GAA	GGG	GAT	GTT	GTG	CAG	TGC	ATC	CGT	GAC	ACG	AGC	AAG
Gly	Cys	Cys	Glu	Gly	Asp	Val	Val	Gln	Cys	Ile	Arg	Asp	Thr	Ser	Lys
			250					255					260		
GTT	ATG	AAC	CAT	ATT	TGT	TCA	AAA	CAA	GAT	TCT	ATC	TCC	AGC	AAA	ATC
Val	Met	Asn	His	Ile	Cys	Ser	Lys	Gln	Asp	Ser	Ile	Ser	Ser	Lys	Ile
		265					270					275			
AAA	GAG	TGC	TGT	GAA	AAG	AAA	ATA	CCA	GAG	CGC	GGC	CAG	TGC	ATA	ATT
Lys	Glu	Cys	Cys	Glu	Lys	Lys	Ile	Pro	Glu	Arg	Gly	Gln	Cys	Ile	Ile
	280					285					290				
AAC	TCA	AAC	AAA	GAT	GAT	AGA	CCA	AAG	GAT	TTA	TCT	CTA	AGA	GAA	GGA
Asn	Ser	Asn	Lys	Asp	Asp	Arg	Pro	Lys	Asp	Leu	Ser	Leu	Arg	Glu	Gly
295					300				305					310	
AAA	TTT	ACT	GAC	AGT	GAA	AAT	GTG	TGT	CAA	GAA	CGA	GAT	GCT	GAC	CCA
Lys	Phe	Thr	Asp	Ser	Glu	Asn	Val	Cys	Gln	Glu	Arg	Asp	Ala	Asp	Pro
			315					320					325		
GAC	ACC	TTC	TTT	GCG	AAG	TTT	ACT	TTT	GAA	TAC	TCA	AGG	AGA	CAT	CCA
Asp	Thr	Phe	Phe	Ala	Lys	Phe	Thr	Phe	Glu	Tyr	Ser	Arg	Arg	His	Pro
			330				335						340		
GAC	CTG	TCT	ATA	CCA	GAG	CTT	TTA	AGA	ATT	GTT	CAA	ATA	TAC	AAA	GAT
Asp	Leu	Ser	Ile	Pro	Glu	Leu	Leu	Arg	Ile	Val	Gln	Ile	Tyr	Lys	Asp
		345				350					355				
CTC	CTG	AGA	AAT	TGC	TGC	AAC	ACA	GAA	AAC	CCT	CCA	GGT	TGT	TAC	CGT
Leu	Leu	Arg	Asn	Cys	Cys	Asn	Thr	Glu	Asn	Pro	Pro	Gly	Cys	Tyr	Arg
		360				365					370				
TAC	GCG	GAA	GAC	AAA	TTT	AAT	GAG	ACA	ACT	GAG	AAA	AGC	CTC	AAG	ATG
Tyr	Ala	Glu	Asp	Lys	Phe	Asn	Glu	Thr	Thr	Glu	Lys	Ser	Leu	Lys	Met
375					380					385					390

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8 / 8
FIG. 4C

GTA CAA CAA GAA TGT AAA CAT TTC CAG AAT TTG GGG AAG GAT GGT TTG
Val Gln Gln Glu Cys Lys His Phe Gln Asn Leu Gly Lys Asp Gly Leu
395 400 405

AAA TAC CAT TAC CTC ATC AGG CTC ACG AAG ATA GCT CCC CAA CTC TCC
Lys Tyr His Tyr Leu Ile Arg Leu Thr Lys Ile Ala Pro Gln Leu Ser
410 415 420

ACT GAA GAA CTG GTG TCT CTT GGC GAG AAA ATG GTG ACA GCT TTC ACT
Thr Glu Glu Leu Val Ser Leu Gly Glu Lys Met Val Thr Ala Phe Thr
425 430 435

ACT TGC TGT ACG CTA AGT GAA GAG TTT GCC TGT GTT GAT AAT TTG GCA
Thr Cys Cys Thr Leu Ser Glu Glu Phe Ala Cys Val Asp Asn Leu Ala
440 445 450

GAT TTA GTT TTT GGA GAG TTA TGT GGA GTA AAT GAA AAT CGA ACT ATC
Asp Leu Val Phe Gly Glu Leu Cys Gly Val Asn Glu Asn Arg Thr Ile
455 460 465 470

AAC CCT GCT GTG GAC CAC TGC TGT AAA ACA AAC TTT GCC TTC AGA AGG
Asn Pro Ala Val Asp His Cys Cys Lys Thr Asn Phe Ala Phe Arg Arg
475 480 485

CCC TGC TTT GAG AGT TTG AAA GCT GAT AAA ACA TAT GTG CCT CCA CCT
Pro Cys Phe Glu Ser Leu Lys Ala Asp Lys Thr Tyr Val Pro Pro Pro
490 495 500

TTC TCT CAA GAT TTA TTT ACC TTT CAC GCA GAC ATG TGT CAA TCT CAG
Phe Ser Gln Asp Leu Phe Thr Phe His Ala Asp Met Cys Gln Ser Gln
505 510 515

AAT GAG GAG CTT CAG AGG AAG ACA GAC AGG TTT CTT GTC AAC TTA GTG
Asn Glu Glu Leu Gln Arg Lys Thr Asp Arg Phe Leu Val Asn Leu Val
520 525 530

AAG CTG AAG CAT GAA CTC ACA GAT GAA GAG CTG CAG TCT TTG TTT ACA
Lys Leu Lys His Glu Leu Thr Asp Glu Glu Leu Gln Ser Leu Phe Thr
535 540 545 550

AAT TTC GCA AAT GTA GTG GAT AAG TGC TGC AAA GCA GAG AGT CCT GAA
Asn Phe Ala Asn Val Val Asp Lys Cys Cys Lys Ala Glu Ser Pro Glu
555 560 565

GTC TGC TTT AAT GAA GAG AGT CCA AAA ATT GGC AAC TGAAGCCAGC
Val Cys Phe Asn Glu Glu Ser Pro Lys Ile Gly Asn
570 575

TGCTGGAGAT ATGTAAAGAA AAAAGCACCA AAGGGAAGGC TTCCTATCTG TGTGGTGATG
AATCGCATTT CCTGAGAACA AAATAAAAGG ATTTTCTGT AACTGTCACC TGAAATAATA
CATTGCAGCA AGCAATAAAC ACAACATTTT GTAAAGTTAA AAA

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/11675

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 A01K67/027 C12N15/00 C12N5/10 C12N1/00
 C12N15/12 C12N15/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS), vol.268, no.11, 15 April 1993, BALTIMORE, MD US pages 8221 - 8229 SIMONET, W.S. ET AL. 'A far-downstream hepatocyte-specific control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice' cited in the application	1-3, 10-12, 19-22, 26-29
Y	see the whole document	4-9, 13-18, 23-29

	-/--	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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 O document referring to an oral disclosure, use, exhibition or other means
 P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

3 February 1995

Date of mailing of the international search report

15 -02- 1995

Name and mailing address of the ISA

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 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+31-70) 340-3016

Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/11675

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS), vol.266, no.14, 15 May 1991, BALTIMORE, MD US pages 8651 - 8654 SIMONET, W.S. ET AL. 'Multiple tissue-specific elements control the apolipoprotein E/C-I gene locus in transgenic mice' cited in the application see the whole document ----	1-29
P,Y	EP,A,0 589 851 (IST. RICHERCHE BIOL. MOLECOLARE ANGELETTI) 30 March 1994 see the whole document ----	1-29
Y	US,A,5 075 229 (HANSON, R.W. ET AL.) 24 December 1991 see the whole document ----	1-29
Y	WO,A,88 10304 (EDISON ANL BIOTE.) 29 December 1988 see the whole document ----	1-29
Y	WO,A,90 13655 (SNOW BRAND MILK PRODUCTS) 15 November 1990 see the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter nal Application No

PCT/US 94/11675

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0589851	30-03-94	CA-A- 2105594	08-03-94
US-A-5075229	24-12-91	AU-A- 1986188	19-01-89
		EP-A- 0365591	02-05-90
		JP-T- 3500482	07-02-91
		WO-A- 8810304	29-12-88
WO-A-8810304	29-12-88	AU-A- 1986188	19-01-89
		EP-A- 0365591	02-05-90
		JP-T- 3500482	07-02-91
		US-A- 5075229	24-12-91
WO-A-9013655	15-11-90	JP-A- 2291267	03-12-90
		AU-B- 630189	22-10-92
		AU-A- 5520390	29-11-90
		EP-A- 0427863	22-05-91
		US-A- 5302698	12-04-94